

Psychiatric illness and freedom

The Soviet Union has predictably resigned from the World Psychiatric Association. The remaining members should put their own houses in order. Prisoners are especially at risk.

THE absence of a Soviet delegation from this year's meeting of the World Psychiatric Association in Vienna (see *Nature* 21 July, p.204) had been well signalled in advance. The Soviet professional association (like those of Bulgaria, Czechoslovakia and Cuba) had resigned instead of risking the ignominy of expulsion. The issue, which has been festering for years, is the conviction of Western psychiatrists that the practice of psychiatry in the Soviet Union has been seriously abused. There is now a wealth of evidence that people in the Soviet Union have been sent to psychiatric hospitals and institutions for treatment when their "illness" has consisted largely of their dissent from Soviet political and social practices. The essence of the World Psychiatric Association's complaint is that forcing psychiatric treatment on people who are not psychiatrically ill is one of the most serious breaches of medical ethics there could be.

Everybody will agree. Forcing treatment of any kind on people who do not need it is ethically as offensive as, say, the participation of physicians in torture. Forcing psychiatric treatment on people is especially insidious because it may often be a self-justifying procedure. Evidence from the Soviet Union that such practices are followed is a sufficient reason why the World Psychiatric Association should have been prepared to expel the Soviet psychiatric association. So will it now suffice that Western participants at Vienna should leave well alone, perhaps repeating at frequent intervals their apparently well-meant declaration that the Soviet and other delegations would be welcomed back into the fold when they have proved that doubtful practices have been abandoned?

Unfortunately not. Plainly a reform of psychiatric practice in the Soviet Union must be a precondition for readmission, but Western psychiatrists should not suppose that their own practices are above reproach. The essence of the complaint against Soviet psychiatry is that people whose personal liberty is already compromised are especially at risk. The obvious difficulty, for psychiatrists in the West, is that most laws on the treatment of psychiatric illness usually allow that patients should for a time be deprived of liberty, locked up against their will. Psychiatrists are often compelled to act as gaolers.

The problem is further complicated by two other issues — the continuing (if now moderated) dispute among psychiatrists in the West about the role of psychotherapy rather than drugs in treatment, and the repeated resurgence of the misguided opinion that psychiatric illness is not so much an objective condition, comparable with, say, hypertension or a broken femur, but one whose definition is to a large extent arbitrary, as much a function of the environment in which a person finds himself as of his state of mind. R.D. Laing is merely one of those who hold to this doctrine of the relativity of psychiatric illness, now the basis of popular legends such as the film *One flew over the cuckoo's nest* (Fantasy Film Productions, 1975).

The underlying difficulty is that the diagnosis of psychiatric illness is not an objective process. The presence of the signs and symptoms is a necessary but not a sufficient condition for a positive diagnosis. Practising psychiatrists will urge voluntary treatment on patients thought to be ill but will take the drastic step of committing them to a hospital only if life in the community at large is impossible for the patient or his close relatives. Characteristic signs and symptoms must be present, but can often

be recognized only by experienced physicians, who carry a burden of social responsibility which is heavier than in most other fields of medicine. In deciding what treatment a patient needs, social incompatibility alone is neither here nor there.

That is how matters should be arranged, but the prominence of environmental considerations in decisions whether people should be treated compulsorily lends credence both to the extreme Laingian view in the West that there is no such thing as madness but only social incompatibility and support for abuses of psychiatry elsewhere. For if a citizen should be so offended by the way in which he is deprived of liberty by the state that he spends the whole of his energy battling against restrictions which others compliantly accept, is that not technically a manifestation of paranoia or, popularly, of madness? The complaint against Soviet psychiatry is that there have been times when the mismatch between a person and his environment has been taken as a sufficient cause for compulsory treatment.

Before psychiatrists in the West congratulate themselves on having reached the point that such a practice is considered in the true sense wicked, they should give some thought to what happens elsewhere than in the Soviet Union. In Britain, for example, especially since it was accepted twenty years ago that institutionalism itself is bad for people, strenuous efforts have been made to keep people out of psychiatric hospitals. Compulsory admissions have declined dramatically, and people are locked up for much shorter periods than in the past. The reputation of the psychiatrists for fair dealing stems partly from these trends, partly from their professional independence and partly from the knowledge that people locked up against their will have a right of appeal. This is why it is unfortunate that an unwilling patient's first right of appeal against incarceration has been removed, by this year's Mental Health Act, from a mental health tribunal on which lay-people sit to a supposedly independent psychiatrist open (rightly or wrongly) to the charge of putting professional solidarity before the interests of a patient he has not seen before. It seems not to be fully understood that psychiatrists need for the protection of their profession a mechanism of immunity from charges such as these.

The condition of prisoners in British gaols is a more serious challenge to the profession. While there is no evidence that psychiatric treatment is forced on prisoners when they are not medically in need of it, there are puzzling variations in the use of psychotropic drugs from one prison to another which suggests at least a somewhat arbitrary practice by prison physicians (who are employed by the prison service itself). And while there is no evidence that drugs are used to control the behaviour of prisoners, official denials of allegations to that effect will be unconvincing so long as the British prison service remains a closed community of prisoners and prison officers. Both as a matter of social equity and so that prison psychiatrists (and prison physicians in general) may be seen to be above reproach, there is an urgent need that these closed communities should be treated by physicians disinterested in their convenient management. And what goes for Britain is probably also true of most of the other countries whose psychiatric associations remain comfortably members of the World Psychiatric Association. That is why the association should not rest on its laurels but, instead, should tackle the contradictions that persist. □



Beware the dustbowl



THE United States learned its lesson about soil erosion the hard way in the 1930s, when an already-crippled farm economy was dealt the staggering blow of losing millions of acres of once-productive land, its topsoil blown or washed away. In the mean, soil erosion was probably no worse then than now, but in the extreme that time of the dust bowl remains unmatched: the stark photographs by Walker Evans of farms denuded or engulfed, songs by Woody Guthrie of dust storms so dense they blocked out the sun and tales such as in *The Grapes of Wrath* of families uprooted have become evocative parts of the legend of those times. Franklin Roosevelt's New Deal quickly made soil conservation an essential part of its farm reforms. The Soil Conservation Service (SCS), which still provides technical advice to farmers on contour planting, drainage and construction of terraces, ponds and windbreaks, was established; and the Agricultural Stabilization and Conservation Service, which this year will spend \$190 million in cost-sharing programmes with farmers, was created to help carry out that advice. But while the progressiveness of these initiatives puts the policies of other nations to shame, they remain woefully inadequate on at least two counts.

The first is the inevitable concern of congressmen to win their share of goodies for their home districts. Thus even districts with a relatively minor erosion problem get almost as much of the federal government's largesse as the most erosion-prone regions. The more substantial inadequacy is the counteracting effect of virtually every other federal programme making direct payments to farmers. The acreage diversion programmes of the past, which were substantially brought back this year with the payment-in-kind programme, all pay farmers not to grow specific crops, with payment linked to the farmer's "base acreage". Thus the incentive is great to till marginal land, and marginal land is the most erodible and would, in the absence of these perverse incentives, be maintained as permanent pasture.

Marginal land brought into crop production in order to qualify for diversion payments is not exactly going to be the farmer's first choice for expensive conservation measures, either. Even on prime land, conservation makes little economic sense. A careful

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REASONS

Photo: US SCS

study in Iowa has estimated the costs of effective erosion control to be three times the economic benefits — short-term benefits that is. Furthermore, fencerows, terraces, windbreaks, strip-cropping and contour planting are increasingly incompatible with the largest machinery now in use.

Mercifully, a bill now before Congress offers a modest step to counter these pressures. And for once, it uses a stick, not a carrot. Flying in the face of the much-hallowed taboo against dictating specific agricultural practices, it threatens sanctions against any farmer who ploughs up permanent pastureland with erodible soil: all federal assistance, including price supports, crop insurance, loans and disaster payments, would be cut off. The heartening and indeed surprising development is that major farm organizations are supporting the bill. To be sure, the lands at which the bill is aimed are not the subject of much controversy. Everyone agrees that they should not be put to the plough. But the bill breaks new ground by defying the perverse convention that the government must not ask anything in return for what it pays to farmers.

A much tougher fight will come if and when it is proposed to dictate conservation measures on mainstream land. Many farmers and farm groups openly question the SCS figures on erosion, sometimes on doctrinal grounds but also because there are legitimate questions about technical procedures. Thus SCS relies for its estimates of soil erosion on what it calls the Universal Soil Loss Equation, developed several decades ago, which is a highly parameterized formula relating precipitation, soil type, slope, tillage practice and conservation factors to erosion. SCS is now compiling the results of a 1982 survey that sent field workers out to a million separate points around the country to gather new data. But the magnitude of this task does not answer the legitimate doubts surrounding the practice of plugging all of these data into a linear parameterization that fails even to account for the fact that soil lost at one point may simply be washed to another in the same field. To win the larger fight, SCS will have to do a lot of convincing. But the bill now before Congress to halt "sodbusting" is a much-needed foot in the door. □

Empiricism in enterprise

The British Government should work out a coherent policy on innovation.

THE British Government seems bent on making a muddle of its policy on the exploitation of innovations generated with public funds. The explanation is that it appears to have no single policy but only a series of empirically derived stratagems for dealing with separate issues as they separately arise. The formation of the new plant biotechnology company called Agricultural Genetics (see p. 296) is only the latest illustration of the muddle, at least as far as the company's relationship with the Agricultural Research Council is concerned.

The difficulty is plain. The council exists to improve British agricultural technology, to which end it spends £100 million a year. On the whole, the council has been successful. Traditionally, innovations of technique or of breeding stock have filtered through to commercial suppliers without much formality, and an energetic extension service has drummed up custom among farmers. This process, now called technology transfer, has been so successful, and so much to the benefit of farmers, that some ministers in the last government were whispering that the time had come when British farmers should pay for the research carried out on their behalf. Now (and instead?) there is to be a commercial organization with an exclusive right to exploit the council's more interesting innovations in a large part of its field.

The obvious objection is that the council's role in British agriculture will have been profoundly altered without public discussion of any substantial kind. Whatever the council may now say about its determination to continue its support of basic research, its impartiality as a source of technical assistance to British companies in general will have been compromised. This is precisely the danger most to be feared in arrangements between universities and their academics and outside commercial interests. Has anybody seriously considered whether the result will be what the government is always urging — a stronger flow of innovation between laboratories and the generality of industry?

A more particular worry to which the House of Commons must pay close attention is the propriety of an arrangement whereby a small group of investors has acquired an inside track in the exploitation of a stream of applied research supported (for the time being) by public funds. The point may of course be covered by the contract between the council and its new commercial partner, but the contract has been classified (by those concerned) as a commercial secret. Nobody wishes to impede the development of biotechnology in Britain, but there is no means by which taxpayers can tell whether what is now proposed is the best way of arranging for that. While the House of Commons is looking into the matter, it might also enquire further into the arrangement between the Medical Research Council and Celltech, which is known to have a break clause but whose financial terms have not been disclosed. □

Star wars

US Senate urges restraint on space weapons tests

Washington

CONGRESS has begun to flex its muscles to prod the Reagan Administration to resume the stalled negotiations with the Soviet Union on banning weapons from space. The Senate voted unanimously last week to block money for space tests of the first US antisatellite (ASAT) weapon. And the Senate Foreign Relations Committee passed a strongly-worded resolution calling for a ban on all space weapons, including those that might be developed under the President's futuristic plan for a "star wars" defence against ballistic missiles.

The fact that both resolutions have come from the traditionally friendly Senate has jolted the administration. But neither is expected to bring the space weapons programme to a halt. The withholding of test money, proposed by Massachusetts Senator Paul Tsongas, had to be riddled with loopholes to win the support of the Republicans. Thus the administration will be allowed to go ahead with the tests if it can certify that it has tried "in good faith" to resume negotiations, and that the tests are necessary to protect national security.

Proving that the tests are necessary should not be difficult under the relaxed certification rules of the Senate. The ad-

To comply with the Senate resolution, the administration need not actually enter negotiations with the Soviet Union, but must show that it is "willing" to negotiate. The administration has already begun to hint that it is more open-minded than hitherto about the prospects for negotiation. An official of the Arms Control and Disarmament Agency (ACDA) said last week that the administration might accept a Soviet suggestion that a group of scientists from each country should begin to discuss — but not negotiate — some of the issues involved. And the administration has suggested including anti-ballistic missile systems in the continuing Strategic

Largest dinosaur from Surrey



THE spectacular clawbone of a new species of carnivorous dinosaur was revealed last week by Dr Alan Charig and a team from the British Museum (Natural History) in London after the discovery by Mr William Walker, an amateur palaeontologist, of parts of a skeleton in a clay pit in Surrey, southern England, earlier this year.

Dated at 124 million years, from the early Cretaceous, the find is important because remains of carnivorous dinosaurs of this period have been very scarce. When restored it will help to bridge the gap between the more abundant finds from the late Jurassic and late Cretaceous.

Although preliminary assessment suggests that this dinosaur may be similar in general morphology to the carnivorous *Megalosaurus* and *Allosaurus* of the late Jurassic, the huge clawbone is quite distinctive. To what use this was put, however, remains speculation.

Nigel Williams

Arms Reduction Talks (START) in Geneva.

Advocates of an immediate moratorium on the development of space weapons regard most of these moves as cosmetic. Representative Joseph Moakley (Democrat, Massachusetts) delivered two letters to the White House last week arguing that tests of the American ASAT, planned for later this summer, should be cancelled. One letter, signed by 100 members of Congress, says that the new weapon, which can be fired from under the wing of an ordinary F15 fighter, is far more capable than the Soviet system, which is ground-launched and must orbit the Earth at least once before attacking its target.

The second letter, signed by more than 40 scientists and arms control experts, warns that deployment of the American ASAT could pose severe verification problems and preclude later negotiation of a limit on space weapons.

The 1967 outer space treaty prohibits the placing in orbit of "nuclear weapons or any other kinds of weapons of mass destruction". Moreover, the 1963 partial test-ban treaty prohibits nuclear tests in space, while the anti-ballistic missile (ABM) treaty (1972) includes an undertaking not to "develop, test or deploy" ABM systems which are "sea-based, air-based, space-based or mobile land-based".

The administration is meanwhile pressing ahead with plans to develop a space-based defence against nuclear attack. A study team, led by former NASA administrator James Fletcher, is expected to complete an initial feasibility study by October. Supporters of the "star wars" concept appear nevertheless to be deeply divided about how the project should be handled.

Some of these differences surfaced publicly for the first time last week during a bad-tempered Senate debate begun by Senator Malcolm Wallop, a Wyoming Republican with a long-standing belief in the need to establish chemical laser weapons that could destroy incoming ballistic missiles. More than 20 senators voted with Wallop on an amendment to concentrate most research funds for nuclear defence on three existing laser projects — Alpha, Talon Gold and Lode — and place them under the army's ballistic missile defence organization. Unless this was done, Wallop complained, the Reagan initiative would be swallowed up within the Department of Defense bureaucracy and produce endless research but no weapons.

Claiming that the three projects promised workable technology that could be converted rapidly into weapons, Wallop accused presidential science adviser George Keyworth and other "confused and self-contradictory" officials in Washington of being preoccupied with exotic technologies that were years away while ignoring the opportunities to build laser weapons already at an advanced state of development.

Peter David



ministration will be able to argue that since the Soviet Union possesses the world's only operational ASAT system, the United States needs one of its own. It may be harder to convince senators that the United States has tried in good faith to resume the ASAT negotiations broken off by the Carter Administration as a result of the Afghanistan crisis in 1979. In 1981, the Soviet Union presented the United Nations with a draft ban on space weapons, but the Reagan Administration has insisted that negotiations cannot start until the United States is assured that compliance with a treaty can be verified.

Plant biotechnology

UK company's delayed budding

THE other shoe has at last fallen. After more than two years of hesitation and enforced delay, the British Technology Group (BTG) announced this week the formation of a company to exploit agricultural applications of the expertise in plant biotechnology of the Agricultural Research Council (ARC). BTG was the prime mover three years ago in the formation of Celltech, with interests in medical applications of biotechnology, which has a right of first refusal on innovations arising in Medical Research Council research.

BTG's partners in the formation of the Agricultural Genetics Company Limited (AGC) are Ultramar, a company with financial and petroleum interests, and the British and European versions of the venture-capital company called Advent, established last year with a capital sum of £10 million provided by Monsanto, a US chemical manufacturer with strong agricultural interests.

At this stage, AGC's initial capital is a modest £700,000, subscribed by the three partners and the company's chief executive, Dr Roger Gilmour, who has been recruited from Griffith Laboratories, Chicago, to run the new company. Eventually it is intended that AGC should have a total capital of £15 million. Dr Gilmour declined to disclose his personal stake in AGC at the company's launch on Monday.

Details of the contract with ARC were also not disclosed, but AGC will have exclusive rights to exploit ARC-supported innovations in non-conventional plant breeding, microbial inoculants and biological control products. One envious investor, not among the initial shareholders, says that the first of these items is a kind of "catch-all". ARC will be recompensed by means of a royalty.

Acknowledging that lead times to bring entirely new biotechnology products to commercial exploitation are likely to be in the range of 5–10 years, AGC will at first act as a technology transfer company and will develop joint ventures with established companies, but it hopes that some products will reach the market in less than two years.

Dr Ralph Riley, secretary of ARC, emphasized this week that the council will continue to support research in the national interest, although it may also undertake AGC-commissioned research in its own institutes.

In answer to criticisms that British publicly-funded research results may, through cooperative agreements, be available to foreign companies before other British companies, Dr Riley said that steps have been taken to ensure that this will not happen. ARC-supported scientists will not be in any substantially different position, but scientific publications of commercial interest and external research consultancies will have to be individually

negotiated through AGC. Fears that overseas companies such as Monsanto, the largest single shareholder in Advent Technology plc and Advent Eurofund Ltd, would benefit unfairly from ARC-supported research could therefore be discounted. ARC scientists will earn royalties from AGC if their work is taken up.

Despite the conspicuous *caveats* about long lead times that can be expected, recent results — such as a recently published method of introducing a dominant selectable marker into transformed plant cells (*Nature* 304, 184; 1983) — give grounds for optimism. The British Technology Group is delighted with the interest now being shown in Celltech and hopes for similar success with AGC. AGC's first products are likely to include ideas developed by New Plant Products Limited, set up by the British Technology Group to support applied research while negotiations for AGC were in progress. **Tim Beardsley**

Prutec looks overseas

In an echo of a dignified complaint by the merchant bank N.M. Rothschild, the British venture capital fund called Prutec, an offshoot of the Prudential Assurance Company, said this week that opportunities for investment in British biotechnology were fewer than it would have wished. The occasion was the announcement that the fund is to invest \$2.2 million in two United States companies, Applied Biotechnology and Biosearch

Prutec policy hitherto has been to invest in British companies or in the development of innovations that have not made necessary the formation of a company. Prutec's field of interest is catholic, but biotechnology has been the mainstay of its business in the past few years.

Applied Biotechnology, founded principally by people from the Massachusetts Institute of Technology and the Harvard Medical School, aims to concentrate on animal vaccines and on medical diagnostics.

Prutec's difficulty in finding British innovations in biotechnology is likely to carry some weight with the British Government, which has been brooding about the future of the British Technology Group (BTG) for the past 18 months. BTG is an informal amalgam of two publicly supported venture funds, the National Enterprise Board and the National Research Development Corporation. It seems already to have been agreed that BTG should lose its prescriptive right to refuse all innovations generated with public funds in Britain, but more recently there has been talk that the rise of venture capital organizations in Britain might make it possible to put BTG to sleep. □

Hungarian biotechnology

Priority plan preempted

THE Hungarian Government — in particular, the ministries of industry, agriculture and health — is anxious to launch a priority programme in biotechnology. The initiative is not, however, well-timed. Industrial managers say that the initiative has come too late, while the scientists do not seem particularly keen to be made responsible for what they see primarily as applied technology.

Tentative proposals were made by the government last autumn, just when the scientific establishment was smarting from major budget cutbacks suddenly imposed during the summer vacation. Even those who admit that cuts were inevitable in the present economic situation resent the manner in which they were made. The across-the-board sweep reduced or even nullified the significance of much research in progress. It would have been far better, and more economical in the long run, some scientists felt, if they had been allowed at least to advise where economies should have been made.

Scientists — and in particular the Academy of Sciences — have responded in this mood to the news that the government wants them to take a leading role in the new "biotechnology" programme. Hungary already has some 16 such programmes. And what the politicians understand by "biotechnology" is difficult to determine — the first draft of the plan seems to have covered a wide range of topics from genetic engineering to animal feed concentrates.

Hungarian scientists are not elitists and many of them supplement their work in basic science by taking on contract research for industry and agriculture. Their objection to the proposed programme was that it was too unwieldy and incoherent and that, in many cases, the relevant theoretical work had already been done, so that the skills of top research scientists would be wasted if they were put in charge.

This misunderstanding has now been ironed out, and it is now informally agreed that the new biotechnology programme should contain not more than five or six projects and that the role of the scientists should be confined to research.

For industry, however, which will be concerned with the practical side of the programme, the choice has, to a large extent, already been made. Hungary's industrial research support system allows every enterprise the choice of ploughing back a fixed proportion of its profits into in-house research or else paying the same sum into a central research pool. In practice, most large concerns prefer to do their own research and several pharmaceutical and chemical enterprises are already well launched in biotechnology.

Vera Rich

Hepatitis-B vaccine

Tempers fray as production nears

AN international row over hepatitis-B vaccine erupted last week, and rebounded badly on the French producer, Institut Pasteur Production (IPP).

To begin with, the French accused the World Health Organization (WHO) of being partisan, favouring a US vaccine over the French. WHO officials vehemently denied the accusation, describing it as a "smoke-screen" to deflect criticism from IPP and saying that IPP "broke its promise" to its customers (and WHO) by using undeclared (American) sources of plasma in the manufacture of its vaccine (see *Nature* 14 July, p.104).

WHO says there has been "silence" from France over IPP's production and testing methods, whereas the US producer (Merck, Sharp and Dohme) has provided full details of protocols, clinical studies and tests done by the independent Center for Disease Control at Atlanta. Although IPP has now promised full details, and although the French vaccine is probably at least as safe as the American, the whole

episode reflects badly on the company.

There may also be commercial consequences. WHO sets general criteria governing any new vaccine to be exported from one country to another. The criteria governing production standards and so on are not mandatory, but 130 of the 160 or so member states are poor countries with little or no capacity to set their own standards and which rely on WHO criteria. It is then up to companies marketing vaccines to declare whether their vaccines meet WHO criteria unless a member state asks for advice on a particular vaccine. No country has yet done this for the French hepatitis-B vaccine.

If IPP has cause for complaint, it may be that it was not involved early enough in the setting up of the WHO guidelines for hepatitis-B vaccine, so the guidelines — first established in 1979 — were better attuned to the Merck process than to the French. Merck, says WHO, was the only company in 1979 consistently producing acceptable vaccine ("ten batches out of ten") which had also submitted the vaccine to independent tests. Plainly it rankles in France, where the present vaccine was originally devised, that Merck had stolen such a march by 1979. But IPP will have a hand, with other producers, in producing the third version of the guidelines due by December.

IPP's "broken promise" seems to centre on an undertaking not to use blood plasma containing the possibly infectious E antigen of hepatitis-B virus (HB-E). Although WHO advisers had not insisted on this requirement, fearing that too little plasma would then be available, IPP's use of US plasma originally unscreened for HB-E and perhaps even found to be contaminated has invalidated the producer's claim to purity, a question on which the scare over acquired immune deficiency syndrome (AIDS) has made WHO especially sensitive.

Meanwhile, however, the fuss may be overtaken by events. The Swiss-based company Biogen has succeeded in producing test quantities of hepatitis-B vaccine from antigen genes cloned in yeast, and at least one other company is hard on its heels. According to Professor K. Murray of the University of Edinburgh, a Biogen adviser who cloned the original gene, human clinical trials may take place by the end of the year.

The technical problem of scale-up "would not be the limiting factor" said Murray but, rather, the time it took for the vaccine to satisfy national regulations for new vaccines. It could be on the market in two years, he said, as a product with no risk of contamination by AIDS or any other infectious agent. "It's an interesting reversal of the fears about genetic engineering of a few years ago" said Murray. **Robert Walgate**

Electron accelerators

Argonne concedes defeat

Washington

THE wrangle between Argonne National Laboratory and a consortium of south-eastern universities over the right to build a proposed new electron accelerator ended abruptly last week with Argonne bowing out of the fight. Saying that stalemate could result in delay or outright cancellation of the project, Argonne director Walter Massey asked the Illinois congressional delegation to call off its effort to overturn the recommendation of an expert advisory committee in favour of the south-eastern group.

The following day, the Department of Energy announced that the proposal from the Southeastern Universities Research Association (SURA) had been officially adopted and would be a candidate for the department's budget request for fiscal year 1985.

Argonne's decision to contest the recommendation of the Nuclear Science Advisory Committee (see *Nature* 7 July, p.7) went against a long tradition of respecting such decisions as the final word of the physics community. Many physicists expressed concern that Argonne's action threatened to drag the system of peer review on technical merits down to the level of pork-barrel politics.

White House science adviser George Keyworth expressed similar concern to Energy Secretary Donald Hodel and to Massey, and was the source of the scarcely veiled threat that if the dispute continued, the simplest solution might be to scrap the project altogether.

Massey said that while the loss of the project was a setback, Argonne would remain "one of the premier laboratories in nuclear physics" on the strength of its ATLAS heavy-ion accelerator, to be completed next year. Argonne has also already proposed another project on the scale of the electron accelerator, an advanced neutron source that would build on the laboratory's experience with its recently-completed Intense Pulsed Neutron Source. A letter to Massey from Illinois Senator Charles Percy last week mentioned continued efforts on the part of the congressional delegation to "locate new projects at the laboratory that equal or exceed the benefits to you and the region that were represented by the accelerator".

If all goes according to plan, and if Congress approves, construction of the 4-GeV accelerator will begin in October 1984, the beginning of the 1985 fiscal year. SURA is requesting \$4-5 million in operating funds for fiscal year 1984 in order to hire a core staff of 35-40 accelerator physicists, engineers and managers and to start essential prototype work. **Stephen Budiansky**

Biogen's inside track

The Hague

EFFICIENCY, safety and cheapness are the main advantages of Biogen's new hepatitis-B vaccine, according to Dr Kenneth Murray's presentation of his findings here last week. The antigen is the result of six years of research in which the partners have been Biogen, the Dutch technological research organization TNO and Dr Murray's unit at the University of Edinburgh.

The Biogen vaccine is an antigen made from the gene for hepatitis-B surface antigen. Dr Murray explained last week that after experiments had shown that the gene could not be expressed in *Escherichia coli*, the team incorporated a sequence simulating the real gene in yeast.

The material is still on trial, but Dr Walter Gilbert, Biogen's chairman, announced last week that two chimpanzees had been successfully immunized against hepatitis B using an experimental vaccine made from surface antigens produced by genetically engineered yeast. Two other chimpanzees, which did not receive the vaccine, contracted the disease after all four animals had been exposed to it. Blood tests confirmed that the chimpanzees receiving the Biogen vaccine were immune.

Biogen hopes to begin tests with its vaccine in human volunteers at the end of this year and hopes for official certification by 1984 or 1985. The potential of the new product is considerable, not least in its relative cheapness. Last week, people were guessing that a dose of Biogen vaccine will cost \$10, compared with an estimated \$100 for the Merck and Institut Pasteur Production vaccines.

Geert Linnebank

French budget

More means less for research

ROUGH estimates are emerging in Paris of the budget that the ministry of research and industry may enjoy next year. The expectation is that the budget will be 7 per cent up on 1983, in current francs, compared with official inflation of around 8 per cent. So the outlook is for a constant budget, more or less — except that true inflation is probably more like 10–11 per cent than the official estimates and that the slow slide of the franc against the dollar (and the mark and the Swiss franc) has increased the cost of imported materials and equipment and of French subscriptions to international laboratories.

Whatever the final version, the real significance of this budget is that the policies established by the previous research minister, and in particular the promise of a 17.8 per cent real rise in budgets each year, is no longer possible. In the months ahead, hard choices will be necessary and it is not yet clear what mechanisms there are to make them. The winners will probably be those fields which have the strongest lobbies at the ministry — biotechnology for example. What worries some in Paris is that there are too few lobbies for the system to arrive naturally at balanced policies,

which sometimes happens in Washington.

At the Centre National de la Recherche Scientifique (CNRS), the largest basic research agency in France, which is supported through the ministry, director-general Pierre Papon hopes above all to hang on to the promised new posts for researchers. A 4.5 per cent increase each year had been planned under Jean-Pierre Chevènement, the previous research minister. "A few weeks ago, we were frightened we might get none", he said. "Now I am almost reassured." CNRS will not get its 4.5 per cent but it will at least get something.

Meanwhile, Papon is brooding about the problem of constant budgets. The best hope is that CNRS may get more than the overall 7 per cent when the ministry cake is divided. Otherwise, "we shall have to make cuts". CNRS priorities (not in order) are: life sciences, humanities and social sciences and the engineering sciences. And although high-energy physics is not a "top priority", Papon would be reluctant to see that field cut. This small mercy should be good news for the high-energy physicists, who are supported by CNRS through the Institut National de Physique Nucléaire et Physique des Particules, whimsically called IN_2P_3 . That enjoyed none of Jean-Pierre Chevènement's largesse and in fact suffered a cut in real terms this year.

Robert Walgate

Computers in France

Beggars must buy Bull

FRENCH high-energy physicists are "beggars in Hamburg and beggars at CERN" for large-scale computing, according to Professor Pierre Falk-Variand, scientific adviser to the director of the Institut National de Physique Nucléaire et Physique des Particules (IN_2P_3). The institute, financed by the Centre National de la Recherche Scientifique (CNRS), manages high-energy and nuclear physics in France.

"I am ashamed of my country in computing", Falk-Variand said in commenting on a recent report by an IN_2P_3 working group which showed that French physicists in these fields have access to "six units" of computing power compared with 30 units for their German colleagues and 24 units for the British. (The IN_2P_3 unit is based on the power of a single CDC 6600 computer and represents the operation of some 1.5–2 million instructions per second.) In the same terms, CERN (the European Organization for Nuclear Research) alone had 19 units of computer power in 1982.

Even in mini-computers, French physicists are falling behind, says the report. This state of affairs has a serious impact on international collaborations, which are now almost inevitable in high-energy physics experiments. "We fought like mad for 18 months to buy a VAX [built by the Digital Equipment Corporation]. When one of my collaborators wants a VAX, he simply signs an order. Here, after 18 months of asking for nine, we got two" says Falk-Variand. It is thus becoming increasingly difficult for French high-energy physicists to play their proper part in data processing in European experiments, even though it is only through data processing that the results of experiments emerge.

The problems facing the French physicists are not financial but commercial. The French Government seeks to help the national computer industry, now all gathered under one banner in the holding company "Bull". However, the physicists usually prefer American DEC, CDC and

IBM machines to French-built computers. Their collaborators use them and vast amounts of software, much of it developed at CERN, is freely available.

To change now to Bull machines would involve a tremendous waste of time, the physicists say. But it is beginning to seem as if they might have done better to write the new software, for the French Government has dug in its heels.

Strangely enough, the French Government has achieved this stranglehold on high-energy physicists without having an explicit policy to divert orders to Bull. The policy directive in force some years ago has lapsed in favour of "free competition", a Bull executive explained last week. But he added that it does not follow that individuals in government departments handling applications for computers "may not favour Bull".

At the Ministry of Industry and Research, where the Mission de l'Informatique holds the real power by exercising a veto over computer purchases out of the public purse, the whole issue is now described as "very sensitive". A new policy is being formulated to plan the development of the Bull group but which should also appease the physicists and other academic users.

One possible solution suggested by Professor Pierre Papon, director-general of CNRS, to M. Laurent Fabius, minister of industry and research, is that CNRS and IN_2P_3 computer specialists should play a greater role in Bull's research and development. This may be seen as a kind of *quid pro quo* for the right to buy the American computers that the physicists (and others) need now, and could improve Bull's market place in the long term.

Papon believes this scheme strikes a chord at Bull. The company's four-year plan, agreed earlier this year, already foresees a much greater degree of cooperation than hitherto between Bull and French researchers.

Robert Walgate

Europe follows US

Brussels

THE Belgian Biochemical Society (SBB), this year responsible for organizing the fifteenth meeting of the Federation of European Biochemical Societies (FEBS) on 24–29 July, is in that delicate state of having arranged a party but of not knowing how well it will succeed. Although small by the standards of the now biennial meeting of the Federation of American Societies for Experimental Biology (FASEB), the FEBS meeting will probably attract 2,000 participants.

The organizers say they have tried to streamline this year's programme in the interests of direct communication between participants. One innovation is a job-placement bureau along the lines of those accompanying major meetings of US societies. Professor Gisele Preaux of the University of Leuven, secretary of SBB, says that interest in the job-placement service has been greater than expected and that there is a plan to provide a permanent information system on the job market.

This year's FEBS meeting will have 200 invited speakers, including seven plenary addresses by Arthur Kornberg (Stanford), Christian de Duve (Rockefeller University), Zouhair Atassi (Mayo Clinic), Piet Borst (Amsterdam), Charles Weissmann (Zurich), Klaus Weber (Max Planck, Göttingen) and Aaron Klug (Cambridge). There will be 1,300 poster exhibits.

Geert Linnebank

Asbestos risks

Manville tries to pass the buck

Washington

MANVILLE Corporation, the asbestos producer that last summer filed for bankruptcy in a pre-emptive bid to shield itself from thousands of liability suits, last week took another step to cut its losses by suing the federal government to recover \$1 million it has already had to pay out to wartime shipyard workers who suffered health damage from asbestos exposure.

The unusual lawsuit accuses the government of failing to enforce its own safety standards at shipyards during the Second World War and of breaching an implied contract to indemnify Manville against liability claims.

Roughly half of the 20,000 claims already filed against Manville involve shipyard workers; if Manville's suit succeeds, it could ultimately shift tens of thousands of millions of dollars in claims onto the government. Manville had settled some 3,000 of these claims before its bankruptcy declaration effectively prevented further settlements.

The lawsuit against the government relies heavily on documents — some only recently declassified — which show that the Navy and the Maritime Commission were aware of excess asbestos exposure among shipyard workers. A 1941 memorandum from the naval commander in charge of preventive medicine stated, for instance, "We are having a considerable amount of work done in asbestos and from my observations I am certain we are not protecting the men as we should. This is a matter of official report from several of our Navy Yards." Other health inspections at the time found as much as five times the limit for asbestos dust then recommended by the US Public Health Service (5 million particles per cubic foot) and reported a continuing failure to improve ventilation and dust collection.

Manville is hoping to broaden the government's liability to include not only Navy Yards but private shipyards as well; the government, Manville says, "effectively assumed control over the shipbuilding industry" by issuing mandatory contracts and orders that swelled shipyard employment from the pre-war level of 168,000 to a peak of 1.5 million. During that time, 6,000 merchant ships were built and the naval fleet was expanded sixfold. Asbestos was used extensively in all the ships to provide lightweight fireproofing and insulation.

The government has recognized workers' compensation claims from employees of government-owned shipyards, but not private yards. A number of recipients of these minimal compensation payments have also sued Manville successfully for more substantial compensation.

Although Manville's suit has brought to light apparently damning evidence of government neglect, that evidence alone is

not a sufficient ground for its case. The government as a rule is immune from liability for its policy decisions; the Court of Claims, where the suit was filed, accepts only cases that claim breach of contract by the government. Manville is thus claiming that because it was compelled by law to accept government contracts and give them first priority as part of the war effort, the government assumed an implied contract to hold the company harmless for the consequences. Moreover, the government, Manville says, effectively took control of the asbestos industry by importing asbestos fibre and directing its allocation to processors.

Assistant Attorney General Paul McGrath last week issued a stinging rebuttal to that claim. "Manville, as a publicly owned corporation, was in the business for

the profit of its shareholders and was not a public service organization", he said.

According to evidence produced in the course of many of the successful liability claims against Manville, the company deliberately kept secret from workers and the public information on the dangers of asbestos. The Public Health Service standard addressed only asbestosis, the emphysema-like condition that affects those chronically exposed to asbestos fibres. None of the government documents referred to by Manville suggests that the government knew of the more serious carcinogenic effects of asbestos. Yet according to plaintiffs in the liability cases, Manville was aware as early as the 1930s that its own workers were developing lung cancer from asbestos exposure. Depositions obtained from former Manville employees acknowledged a deliberate, so-called "hush-hush policy" among Manville's management at that time.

Stephen Budiansky

Polish salt-mines

Zlotys for church conservation

THE Polish Government has raised the price of edible salt to between 11 zloty and 17 zloty per kilo (depending on type and packaging). The increase is not directly related to the current economic crisis but rather to the need to establish a national fund for one of the country's leading historical and artistic treasures — the salt mine at Wieliczka, near Krakow.

Salt has been mined at Wieliczka since the thirteenth century, and its production still accounts for 40 per cent of Poland's output. The major part of the mine — which in all has more than 200 km of galleries on nine levels and a maximum depth of 330 m — has become a tourist

attraction, which takes the visitor through grottoes showing legendary and historical scenes, a museum of early mining equipment, the horse-drawn imperial tramcar used for the visit of the Austrian emperor Franz Josef to the mine and even a full-sized church, all carved out of the rock. In 1978, the excavation was listed by Unesco in its world cultural heritage file.

Salt-bearing strata — known as "green salt" in Poland — are not, however, the most durable medium for sculpture, and for some years, the state of the carvings has been causing considerable concern. An expert team from the Krakow Mining and Metallurgical Academy (AGH) put forward a number of proposals for averting further damage but little if anything seems to have been done to implement them.

A conservation programme was formally inaugurated in 1960 but it was not until 1979 that the Mining Committee of the Polish Academy of Sciences produced a detailed report on the state of the excavations. This led to no fewer than eight government resolutions but still no action. By last August, the Party daily *Trybuna Ludu* admitted that it would no longer be feasible to save more than the grottoes of exceptional historical and artistic interest, and that even that would require three years for "technical preparations" and at least 20 years for the conservation work.

Faced with the total lack of "means, technicians and institutional support", two public-interest groups, one in Krakow and one in Warsaw, last summer launched an appeal fund. How much money they managed to raise is unknown; the new salt-surcharge, however, suggests that the Polish Government is at last prepared to give some practical support to the scheme.

Vera Rich

Canadian MRC

Washington

CANADIAN medical researchers are breathing more easily now that the government has restored cuts in the Medical Research Council (MRC)'s budget that had been called "disastrous" by prominent members of the research community there (see *Nature* 16 June, p.561).

Just before the start of the fiscal year on 1 July, the government granted MRC an additional \$50 million over two years beyond the \$117 million already approved. The extra money enabled MRC to award an additional 221 research grants, 43 fellowships, and 61 grants that had been deferred for as long as two years. The supplement effectively brought the award rates for grants and fellowships up to the level of past years. In addition, MRC was able to award two new biotechnology training grants, "recognizing Canada's urgent need for qualified investigators in this area," the council said.

Stephen Budiansky

UK research

Council considers economies

THE UK Science and Engineering Research Council (SERC) has decided to present a constructive and positive response to the Rayner report on its activities, despite criticisms of the report by SERC staff. Although the council disagrees with one of the report's chief recommendations, it will nevertheless investigate over the next few months the recommended sale of Herstmonceux Castle at the Royal Greenwich Observatory (RGO) and the possible merger of RGO with another SERC establishment.

Most of the recommendations of the Rayner report (see *Nature* 7 July, p.3) affected activities peripheral to scientific research. Three of its more significant proposals, however, provoked strong criticism from the staff side (not to be confused with the council itself).

These proposals are (a) that the Rutherford Appleton Laboratory should sell off 95 houses that it owns and uses to encourage staff recruitment; (b) that Herstmonceux Castle, a major component of RGO, should be sold and replaced by a new extension to other buildings on the site; and (c) that the implications of the merger of RGO with another SERC establishment (perhaps the Royal Observatory, Edinburgh) should be examined before April 1984. The first two recommendations, according to the report, would save SERC £3.3 million in capital and £430,000 a year in running costs.

The sale and the merger proposals provoked the strongest protests from SERC staff on the grounds that they have scientific consequences outside the Rayner unit's remit and competence. The SERC council has, however, decided to consider both proposals, partly because it seems politically sensible to do so (the Rayner unit was set up at the instigation of the Prime Minister, Mrs Margaret Thatcher) and partly because of the shortage of funds brought on by the increase in the sterling cost of subscriptions to overseas research organizations.

The council, which endorsed many of the other recommendations, disagreed last week with the proposed sale of houses at the Rutherford Appleton Laboratory on the grounds that their rents and costs balance and that they are a significant aid in recruiting staff.

The council's chairman, Dr John Kingman, will next present the SERC management response to the Secretary of State for Education and Science, Sir Keith Joseph, who will decide what action is to be taken. The period between now and next spring seems likely to be an anxious one for SERC. The Advisory Board for the Research Councils (ABRC) is working on its own recommendations for the pattern of science spending over the next financial year. Second, a Treasury committee is

investigating whether SERC can carry forward to subsequent financial years some or all of its outstanding budgetary excess or deficit; a positive recommendation might release SERC from the severe financial pressures it faces at present. Third, however the Secretary of State for Education and Science responds to the ABRC recommendations, the British Cabinet's decision to reduce projected public spending by £5,000 million in 1984-85 may yet force the Prime Minister to abandon her promise that scientific research would be "protected".

Philip Campbell

Scientists in India

From a correspondent
New Delhi

ABOUT 290,000 scientists and engineers, 15 per cent of India's total scientific and technological manpower, are unemployed, according to statistics recently released by the Department of Science and Technology (DST). The number of jobless is expected to rise to 373,000 by 1985, when the stock of scientific manpower is predicted at 2.47 million, an increase of 25 per cent over the present 1.95 million. Although unemployment continues to worsen, universities are turning out 37,000 scientists and engineers each year, 3,000 of them PhDs.

One fact that stands out from the DST report is that the scientific manpower figure is no index of the country's research and development strength. Nine out of ten scientists and engineers are not employed in any activity related to research and development. According to the statistics, only 184,000 are actually employed in research and development institutions, of whom 64 per cent are working in administrative and non-technical positions. In other words, the effective manpower is just 60,000, which gives the lie to the often quoted statement that India has the world's third largest scientific and technical manpower.

Other highlights of the statistics for 1980-81 (the latest year for which figures are available) are as follows.

- India spent about £500 million (0.66 per cent of gross national product) on research and development in 1980-81 (a figure that rose to about £660 million in 1982-83). Three-quarters was spent on applied and experimental research, 16 per cent on basic research and 8 per cent on other activities.
- Fourteen per cent of the total came from the private sector, 8 per cent from state governments and 78 per cent from central government. The 470 private in-house research and development units and 62 units in the public sector spent about £130 million on research and development.
- Of the scientific and technical personnel engaged in research and development, 11 per cent were PhDs.

Polish education

New guise for martial law

POLAND'S new special powers act, seen by many observers as little more than martial law in civilian dress, contains several clauses which cut across the principles of academic self-government embodied in last year's Higher Education Act. In particular, the new provisions offer the government the following opportunities.

● State "administrative organs" are granted, as a "temporary" measure, the right to enter the "sphere of activity" of universities and colleges to restore "disturbed legal order".

● The Prime Minister has the right to abrogate resolutions of the Main Council for Science and Higher Education and of the academic councils of universities should these prove to be "incompatible" with the law or with "vital social interest".

● On similar grounds, the academic councils and other collegiate organs of universities and colleges may be suspended for not more than six months, rectors and deans may be dismissed and replacements appointed.

● Students and lecturers may be suspended temporarily if they commit actions which are "particularly harmful" in social terms or aimed against the vital interests of the Polish People's Republic. (The inclusion of the word "particularly" is apparently a concession to the Catholic lobby within the Sejm.)

How far the amnesty for activists will go is not yet clear. It is known, however, that the six intellectual "advisers" to Solidarity and the leading Solidarity activists now awaiting trial will not be included. As far as persons in hiding or abroad are concerned, the amnesty is dependent on former activists giving a full account to the police of all their activities and, in particular, a list of their associates. It is not clear, therefore, how many will feel that they can, in conscience, take advantage of the offer.

Students stranded in Britain by the declaration of martial law, many of whom had been active in the now-banned Independent Students' Association (NZS) and who have found places in British universities under the auspices of the Polish Students' Appeal Fund organized from the London School of Economics, will be staying on in Britain.

One feature of the lifting of martial law, which had been expected for some weeks to be timed for the national day of the Polish People's Republic on 22 July, was the rigour with which its measures were applied right up to the end. Even a few days earlier, scientists wishing to travel abroad to conferences were being told that visas would be granted only if they formally renounced such "anti-state" activities as having signed a petition for an interned or imprisoned colleague.

Vera Rich

Embryo sexing

Cattle now, people next?

DR ROBERT EDWARDS, who with Mr Patrick Steptoe pioneered *in vitro* fertilization techniques in the treatment of human infertility, has high hopes that recent advances in molecular biology will soon provide a means of sexing human embryos before implantation in the uterus. This, he says, would be a first step towards an effective programme for the treatment of inherited diseases.

Rabbit embryos have been sexed and reimplanted by excising a piece of trophoblast at a comparatively late stage of embryological development, but the small size of human embryos makes this approach appear difficult if embryos are to be successfully reimplanted. In collaboration with Dr Jack Cohen, Dr Carol Fehilly and Dr Simon Fishel, Dr Edwards hopes shortly to start using DNA probes to identify human embryo cells containing the Y chromosome; however, this work will not begin until the ethical position in Britain has been clarified. The isolation of single copy DNA sequences specific for the human Y chromosome was reported in *Nature* last month (303, 831; 1983).

The technique would involve dividing embryos at around the eight-cell stage. One half would be sexed by autoradiography with labelled DNA while the other would be frozen for reimplantation in a subsequent cycle. The portion of the Y-chromosome determining sex in sex-reversed mice has been identified in single

cells by Dr Lalji Singh and Dr Kenneth Jones, at the University of Edinburgh. Their test, which used satellite DNA from the W chromosome of snakes, is unlikely to be applicable to human cells, but the principle has been shown to be workable.

By looking for sex-specific DNA sequences directly, problems over gene expression in early embryos are avoided. Later it may be possible to develop probes for other gene loci.

However, Genetic Engineering Inc., of Denver, Colorado, is already offering sexed cattle embryos for reimplantation. Rather than looking for DNA sequences, their test looks for a gene product expressed on the cell surface. The company will say of its test, for which a patent application has been applied, only that it does not in-

volve removing cells from the embryo and that it is based on a monoclonal antibody specific for the H-Y transplantation antigen. The technique is said to be quicker and cheaper than methods based on the karyotype of trophoblast cells.

The service has been offered in the United States since April, and the company is claiming an 80 per cent success rate for its test. The rate of successful reimplantation is no lower than is normal for embryo transfer — around 60 per cent. Furthermore, the company says, embryos can be sexed in groups, at around the 64-cell stage. Dr Edwards describes Genetic Engineering's results as "extremely interesting". The test is at present available only in the United States; it is thought that ambitious marketing plans for sexed embryos, which would command a high price from cattle breeders, are being held up by difficulties in making the test usable by those not qualified as embryologists. **Tim Beardsley**

Soviet nuclear energy

Accident at construction plant

SPECULATION continues about what may have been a serious accident at the Atom-mash plant in Volgodonsk, but without much help from Soviet sources. Indeed, the belief that there may have been some trouble at the plant stems only from an apparently routine report in *Pravda* of a meeting of the Volgodonsk city party under the headline *To increase responsibility*.

This format makes the report indistinguishable at first glance from standard "party page" criticisms of poor performance. A careful reading of the article reveals, however, that more was at stake than a failure to meet production targets — the Atom-mash management, says *Pravda*, had "failed to ensure the accident-free exploitation of engineering communications", a convoluted way of saying that some accident had occurred.

This admission is probably a setback for Soviet nuclear energy planners, whose current proposals include a network of twelve nuclear power-cum-district-heating stations to serve the major cities of the western Soviet Union. These stations will be on the outskirts of the cities but sufficiently near to allow their hot water to be used for heating. They will be surrounded by some 2 km of recreational parkland. An alternative scheme proposed a few years ago for large nuclear power complexes safely sited in remote Siberia and feeding the all-union power grid never won a hearing outside the pages of the party theoretical monthly *Kommunist*.

Just what happened at Volgodonsk is not clear, but it was evidently serious enough to merit censure in the weekly politburo meeting of 14 July and to require a site visit from politburo candidate-member Vladimir Dolgikh. A new State Committee to supervise Work Safety in the Atomic

Power Industry has also been established.

Radioactive material is almost certainly not involved in the accident. *Pravda* has stressed that the reactors and reactor components produced at Volgodonsk are not charged on the site but are sent for assembly to power stations throughout the Soviet Union. What will matter is the degree to which the accident interferes with the ambitious building programme.

Volgodonsk is essentially a boom-town, developed for and around the Atom-mash plant which, says *Pravda*, ought to be the "flagship" of the nuclear power industry. Any disaster, or even setback, at such a plant must inevitably have political overtones. Ironically, however, the accident — whatever it entailed — may bring some small benefit to the nuclear engineers by permitting a more open discussion of possible hazards and safety measures.

This has always been possible within the technical journals — last April, for example, the monthly *Atomnaya Energiya* carried several articles on the subject.

Vera Rich

Frozen embryo aborts

Canberra

HOPES for the birth of the first baby developed from an embryo fertilized *in vitro* and stored in a frozen state faded last week when a pregnancy achieved from such techniques ended in miscarriage. A streptococcal infection following rupture of the chorionic membrane led to a still-born birth at 23 weeks. The conceptus, weighing 560 grams, was a girl. Phenotypic and histological studies have shown the tissues to be normal but karyotypic data are not yet available. Professor Carl Wood, whose group was responsible for the *in vitro* fertilization and freezing techniques, believes the miscarriage is unlikely to be linked with the freezing techniques. Dr Alan Trolunson, who implanted the embryo six months ago, was "very disappointed, particularly because we were so close to success". They are pressing on with the embryo freezing programme.

Meanwhile, draft legislation affecting some *in vitro* fertilization procedures is being prepared by the standing committee of attorneys-general (states and federal) following a meeting of attorneys-general at Mackay, Queensland on 15 July.

Vimala Sarma

No cancer risk

A SURVEY of 15,000 workers employed by British Nuclear Fuels, the publicly owned operator of the Sellafield (previously Windscale) nuclear separation plant, has apparently shown that the incidence of fatal cancer is less than in the general population. This conclusion is based on 1,600 mortalities among previous members of the workforce, among whom the occurrence of various radiation-linked cancers was found to be either statistically indistinguishable from the general incidence or significantly less. The survey, the company said, is continuing. □

Publicity and priority

SIR — The Imperial Cancer Research Fund (ICRF) is a charity dependent solely on the goodwill of the public that sustains us and devoted wholly to the cause of research aimed at preventing and curing cancer. We therefore have a responsibility to tell the public what we are doing and what are our achievements in order that they may appreciate the value of the trust they put in us and continue to support us until our mission is eventually accomplished. One important way of reaching the public is to report, through the normal vehicle of a press release and subsequent interviews, significant new results published in the scientific press, as we did for the work of Dr Waterfield and his colleagues on the relationship between the platelet-derived growth factor (PDGF) and the *sis* oncogene. It was my responsibility as director of research to suggest that this work should be the subject of a press release, which I did before I knew of the impending publication of the parallel and independent study in the United States, and to approve the final form of the statement made to the press.

Our reason for bringing forward this release was not, as you say (*Nature* 14 July, p.108), because we "... feared [we] would gain no credit for Waterfield's prior discovery", since we do not believe, as you seem to, that scientific priorities can be established by articles in the daily press. We were specifically asked by BBC Radio to make a public statement about Dr Waterfield's work. Had the story broken in this way to the exclusion of all other media, our news would not have had the impact that work of this importance merits, nor would we have been fulfilling our duty to the public who support us to keep them properly informed. Those of us who sometimes have the responsibility of dealing with the general news media have to be aware that a press release cannot be a long balanced technical statement giving due reference to all the many individuals usually involved in major discoveries that are always built on the work of others. Scientific accuracy must be blended with simplicity, exciting possibilities for the future must be tempered by cautious optimism and not exaggerated promises. All this we did with the utmost caution coupled with extensive further explanation in informal and formal interviews.

Arguments about scientific priority, especially in such cases as this which involve truly independent work arriving at the same discovery, are sterile and disruptive. Simultaneous independent observations only serve to strengthen the validity of the result and emphasize the teamwork and worldwide interplay of scientific activity, such as the exchange of Dr Doolittle's data base, that contribute so fruitfully to current research achievement.

We have long had a policy of providing general resources in a wide range of areas for our scientific work and, in this case, for example, the results of Dr Waterfield and his colleagues depended on a well-established policy of accumulating large DNA and protein sequence data bases and the resources for their analysis as developed by Peter Stockwell, Chris Rawlings and others here at ICRF, a policy established and implemented well before May this year. We emphasize the value of interaction and stimulation between our various scientific groups and, again here, Dr Waterfield and his colleagues derived stimulus from the work of Dr Rozengurt and his colleagues at ICRF on PDGF and other similar growth factors. To explain all of this and the complete background to the work would take more than even your distinguished scientific journal would accept, let alone could be compressed into a short press release.

I sincerely hope that your news item together with your republishing an extract from the *News of the World* does not represent a retreat from your long-established tradition as a major scientific journal, to a policy of publishing science as it might be in the general news media. Otherwise we should lose the benefit of your pages for proper presentation of scientific results where they belong, an activity not to be confused with their presentation to the general public.

WALTER F. BODMER
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Election recount

SIR — If most Cambridge dons do not understand their own system of single transferable votes (*Nature* 16 June, p.568), it is clearly not suitable for ordinary electorates.

There is one system that is fairer than first-past-the-post without either being incomprehensible or leading automatically to coalitions or breaking the link between representative and constituency. This is the two-stage election as practised in France under the Fifth Republic. In the first round, only candidates receiving more than half the vote are elected. Elsewhere there is a play-off between the two leading contenders. Supporters of disappointed candidates are thus able to express their second preference and all voters can adjust their choice in the light of the results of the first round.

It is interesting to speculate on what would have been the result of applying this system in our recent British election: 318 candidates, including 234 Conservatives and 70 Labour, would have been elected in the first round. Thus just over half the constituencies showed a clear preference. In

the second round, Alliance candidates would have faced a Conservative in 57 seats, a Labour candidate in 28 and a minor party in 4. It seems fair to assume that the Alliance would have won most of these seats, being closer to whichever major party had been eliminated in the first round. Their grand total would have tended towards a maximum of 106.

In 219 of the remaining 233 play-offs, Conservative would have faced Labour. Which would have picked up most seats depends which the rest of the electorate considered least extreme. Note, however, that the Conservatives needed only 92 of these seats to win an absolute majority. Labour could not have reached a majority even by winning all of them. It is therefore clear who was the most likely winner.

It has been said that the French system is only suitable where parliament does not choose the prime minister. It looks as though in the present case it would have produced the same government, but with more visible support and with fair representation for the medium-sized parties.

PHILIP J. STEWART
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Oxford, UK

In the beginning . . .

SIR — Your leading article "Embryology needs rules, not new laws" (*Nature* 28 April, p.735) is a striking example of informed self-deception. It claims that the view that early human embryos are alive is "misguided", and gives what it calls a logical argument to justify this. Allow me to take this logic a little further.

The argument is based on the premise that a living thing must be "potentially autonomously self-replicating", and concludes that, since an embryo is not autonomous, it is not alive (we shall, as does your writer, ignore the "potentially").

Having accepted this, let us now take the case of the newly born infant. This child cannot feed itself, it cannot move by itself, it is unable to reproduce and it is therefore, in fact, not an autonomously self-replicating entity (again we ignore "potentially"). We have, as a logical extension of your writer's argument, to accept that this creature is not alive and that we are morally free to use it for any experiment we wish.

We could extend the argument even further to include the mentally handicapped, the physically handicapped, the old or indeed any helpless individual who cannot make his opinion known. The logic may be correct but do we not have *reductio ad absurdum*?

To say that the individual laboratory, not the law of the land, should be able to decide what is permitted is equivalent to saying that it should be left for me to decide whether I should be allowed to murder my wife.

STEVEN LOVELL
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Summer does not spell greenhouse

General curiosity about climatic change is usually provoked by cold winters or hot summers. But speculation is too little constrained by statistics.

IN spite of the attention that has recently been paid to the possible effects of carbon dioxide on the climate, and to anthropogenic influences more generally, this summer has once again confirmed that the most powerful anthropogenic influence on climate as a topic of conversation remains what it has always been — the blend of curiosity and incredulity that makes each annual seasonal alternation a cause of public astonishment tinged with suspicion that the world is about to come to an end.

In parts of Britain and North America, for example, the occurrence during the past few weeks of consecutive days of higher than usual temperatures has triggered off a string of unruly speculations. The opinion that dust from last year's Mexican volcano El Chichón *must* be responsible for this year's good summer is often held in Britain. Fortunately there has so far been only meagre speculation that the cause may be the even more distant behaviour of the El Niño current in the Pacific. For if fluctuations of this current can profoundly affect the weather in the southwestern Pacific up to six months later, may they not also explain why enthusiasts for the British sport of cricket are this year complaining not that it is too wet to play the game but that the grass surfaces are too hard? And atmospheric carbon dioxide is also an obvious explanation.

What follows is a simple and old-fashioned statement of the reasons why none of these explanations is necessary (which implies that it is irrelevant to this year's weather whether one or more of them may be sufficient). The statistical traps in telling what to make of one warm summer are familiar but nevertheless are generally overlooked. Especially in places such as Britain, the mean temperature for, say, July may vary from one year to the next by as much as 5° centigrade. At any one location, evidence of climatic trends is well buried in such noise (but less deeply on the eastern seaboard of North America than in Western Europe). Thus data compiled by J. de Vries (University of California, Berkeley) for the mean winter temperature (December, January and February) in the Netherlands show a standard deviation of 1.98° centigrade on a mean of 2.16°, between 1945 and 1975, which no doubt explains why the canals are not always safe for skating.

In these frustrating circumstances, those looking for trends tend to fall back on running means (5 or 10 year averages of

monthly mean temperature, for example). One snag is that too short a period will leave too much noise but too long a period will obscure a trend. British amateur climatologists should here again be warned that Britain is almost the worst place to look for trends. H.H. Lamb (in his monumental *Climate: Past, Present and Future*, Vol. 2, Methuen, 1977) has compiled 40-year running means of mean July temperature at various places during the past century. The data show that at Toronto, the temperature increased by 1.5° centigrade between 1840 and 1960, but the corresponding figures for Britain show no trend at all but only fluctuations. It is ironical (but probably not accidental) that Britain should be so well endowed with curiosity about climatic change yet so poorly placed to tell the signal from the noise.

Despair about climatic noise has led to other stratagems — in particular, to the search for some climatically related phenomenon that might prove to be a sensitive indicator of underlying trends. Thus Lamb (*op. cit.*) has lovingly collected data indicative of hot summers in central England (defined as a mean temperature of 16° centigrade or more in June, July and August). His chief finding is that there are never more than four of these in a decade (in the 1770s, 1800s and 1830s). Since the restoration of the Stuarts in 1660, there have been two decades with no hot summers, eight with one, ten with two and seven with three. This distribution is more peaked around the mean than a Poisson distribution, but not significantly so. The obvious difficulty is that these warm summers do not fit well with what little is known of global trends — the summer of 1976, the warmest in Britain since 1862, occurred in a cool year for the Northern Hemisphere as a whole.

The moral is that to attempt to identify global trends in climate from records of a simple variable at a single place is like trying to assess the state of the world's economy from the price of caviar at a local supermarket. For one thing, what happens at one place may be negated by what happens elsewhere. Thus Lamb records that cold winters in Britain appear to have been winters in which Lake Suwa in Japan remained unfrozen and *vice versa*.

Swings and roundabouts are thus one obvious source of the annual fluctuations that occupy climatologists. And the heat capacity of the troposphere is only one way in which solar energy may be stored. The

dynamical energy of the atmosphere (including its distribution of mass with altitude) and the energy associated with its chemical composition (the evaporation of water for example) must be in fluctuating equilibrium with sheer atmospheric enthalpy, while energy also flows in each direction between the oceans and the atmosphere. Trend-spotters should more zealously consider all these causes of fluctuation before claiming success.

In all the circumstances, it is simple good luck that there has been some success in picking out a few significant climatic trends. Thus it seems, on the basis of temperatures averaged over longitude, that there was a marked warming trend between 1910 and about 1940, followed by a significant cooling in the succeeding 30 years (see Wigley, T.M.L. and Jones, P.D. *Nature* 292, 205; 1981). These recent trends, however, are far from comforting for the carbon dioxide lobby. The early warming trend had the sign expected of the greenhouse effect, but was much larger than predicted for the 14 per cent increase of carbon dioxide concentration between 1900 and 1940 (although there is some evidence that the equilibrium concentration may have been less than generally assumed, perhaps only 260–270 parts per million). The cooling trend thereafter is, however, frankly inconsistent with the greenhouse model. This is why Wigley and Jones concluded, two years ago, that even if a warming trend due to carbon dioxide has set in since 1970, it will be necessary to wait for a period comparable with that of the early warming trend before being certain what is afoot. Obviously the problem would be simplified if something were known of the causes of these earlier fluctuations. The moral, for those who would demonstrate the reality of the greenhouse effect, is to pay more attention to the other causes of low-frequency climatic variation.

This does not imply that the calculations of the greenhouse effect so far carried out are to be taken at their face value. While only a half of the carbon dioxide released apparently accumulates in the troposphere, doubts must persist about what happens to the rest. Similarly, too little is known of the exchange of heat (or even carbon dioxide) between the atmosphere and the oceans, while most of the numerical climate models so far do not allow for real clouds, or for the patchiness of the Earth's albedo.

John Maddox

Cosmology

Deciphering the cosmic code

from Joseph Silk

TAKE a mixture of gas and dust, cook it appropriately with the aid of a large computer and a galaxy may emerge. That, at least, is the dream of astronomers who study the most remote galaxies. For we see these galaxies as they were many billions of years ago. A youthful galaxy should differ in appearance from a mature galaxy, and an understanding of galactic evolution is crucial to deciphering the observational evidence. In practice, a much more pragmatic approach is adopted. One begins by synthesizing the spectrum of a nearby galaxy, and systematically develops an increasingly sophisticated model which can cope with very distant and possibly younger galaxies. A recent workshop* was largely devoted to modelling galactic spectra.

Stars provide a vital ingredient. C. de Loore (University of Brussels) described how the evolution of massive stars is affected by mass-loss via stellar winds and by convective mixing after their hydrogen fuel is exhausted. Massive stars are indeed observed to be shedding substantial amounts of material at velocities up to several thousands of kilometres per second. Since the rate of evolution of a star depends in a critical way on the gravitational field and central temperature, the implied change in mass can have a large effect on the luminosity and colour of the star as it ages after hydrogen-burning. While the actual mass-loss rates are quite well known, they differ considerably from star to star, even at similar luminosity. The main constraint comes from the Hertzsprung–Russell diagram which interprets the relationship between stellar luminosity and effective temperature in terms of stellar ageing. Unfortunately, the spread in mass-loss rates for stars that are otherwise indistinguishable in the diagram allows considerable freedom in choice of mass-loss and convection parameters, and consequently in stellar enrichment, for example.

But such massive stars have died after some tens of millions of years, their evolution culminating in a fiery supernova explosion. It is stars of lower mass that dominate the light from nearby galaxies, and their properties were reviewed by A. Renzini (University of Bologna) and I. Iben (University of Illinois). As stars exhaust the hydrogen fuel supply in their cores and begin to burn core helium, their outer layers swell up and they become luminous red giants. Eventually mass outflows deplete the outer envelope as the nuclear fuel supply in the stellar core is exhausted and the hot contracting core is exposed as a white dwarf. These processes take ten billion years or longer for stars of solar

mass, and could contribute importantly to the blue and ultraviolet light from galaxies.

The low- and intermediate-mass stars account for some of the enrichment of heavy elements. Such old stars can develop into supernovae if a white dwarf is destabilized by accretion of matter from a close binary companion, and prolific production of iron-peak elements occurs in the ensuing collapse and explosion. The massive stars are important producers of oxygen, and observations of oxygen abundance were reviewed by F. Vilefond (Observatoire de Paris) and B. Pagel (Royal Greenwich Observatory). Surveys of HII regions, the sites of recent massive-star formation, reveal that in the outer regions of our Galaxy and of other galaxies, systematically lower abundances of oxygen relative to hydrogen are found with increasing galactocentric distance. Simultaneously the mean effective temperature of the stars increases. This is due either to an increase in the number of massive stars or to an increase in the mass of the most massive stars present. In principle, both effects could be acting, but Vilefond argued that the latter effect was more important. He also found that the trend continued in extragalactic HII regions, which include the most metal-poor massive-star-forming system hitherto discovered. Pagel demonstrated that the decrease in oxygen abundance could be correlated with the mass surface density of the galactic disc. The oxygen gradient may be generated either by the incorporation of infall into the galactic disc by primitive metal-poor gas, or by a variable nucleosynthetic yield. In our Galaxy, there is little evidence for infall, although this may not be typical for other systems. But it is possible that the stellar yield of processed matter per unit of mass consumed in star formation and remaining in long-lived stars or compact remnants decreases with galactocentric distance or decreasing surface density; this could explain the oxygen gradient.

The dust component has been measured directly in our Galaxy by extinction studies of bright stars. Ultraviolet observations were compared with similar data for the Magellanic Clouds by K. Nandy (Royal Observatory, Edinburgh), who emphasized the weakness of the 2,200 Å graphite feature in these irregular galaxies. Models indicate that in the Large Magellanic Cloud, graphite is depleted relative to the silicate component, known from the 10 µm feature to be a major grain constituent in our Galaxy, by a factor of 3. In the Small Magellanic Cloud, this depletion is by a factor of 8. Translation of the graphite depletion into information about the carbon abundance is difficult, because, as em-

phasized by B. Savage (Wisconsin), formation and destruction mechanisms for interstellar grains are poorly understood. This is still a potentially important result, because the major source of carbon is from low-mass stars, whereas massive stars produce oxygen. Variations in the proportion of high- to low-mass stars are among the most important ingredients to incorporate into models of stellar population synthesis (G. Bruzual, Centro de Investigaciones de Astronomía, Venezuela).

Indeed, Bruzual managed to provide a remarkable synthesis of the spectrum of the light from the centre of the Andromeda galaxy, Messier 31, a region of exclusively old stars. His model incorporates star formation and star death, and can be extrapolated back in time some ten billion years. One or two galaxies, selected by their radio properties, have now been discovered at a redshift larger than one, corresponding to a look-back time of some 10 billion years. Bruzual found that the colours of these remote objects were bluer than those of nearby ellipticals, even when suitable allowance was made for the reddening due to the expansion of the Universe. He interprets this as a sign of spectral evolution. The amount of evolution is modest, but nevertheless signifies star formation in these ellipticals over the first ten billion years of their history. This long period of star formation came as a surprise, since most cosmologists had expected star formation to last no longer than a free-fall time, some hundreds of million years, in an elliptical galaxy.

Some notes of caution, however, were sounded. Radio galaxies are often very luminous galaxies, and may have evolved quite differently from more normal galaxies. S. Lilly (ROE) noted that the infrared colours of the most distant radio galaxies, obtained at the UKIRT telescope, revealed little or no evidence for evolution. This result has the potentially exciting implication that radio galaxies may provide standard candles for use in cosmology, and infrared measurements may lead to a new determination of the cosmological deceleration parameter. Evolutionary effects wreak such havoc with the interpretation of optical data on the redshifts and magnitudes of remote galaxies that a solution does not seem to be forthcoming soon unless a radical new approach is opened up (R. Kron, Chicago). The IR spectral region could provide such an approach.

Another source of uncertainty in interpreting the optical observations of distant galaxies is that the ultraviolet spectra of nearby ellipticals display considerable variation. Most are flat, indicating few very hot stars; but one or two, including the giant radio galaxy Messier 87, show an increase towards the far ultraviolet (R. Ellis, Durham). Whether this blue light is due to a few newly formed massive stars, as proposed by P. Gondhalekar (Rutherford), or to an old evolved population of low-mass stars, is uncertain. This uncertainty has considerable implications for

*A workshop on 'Spectral evolution of galaxies' was held at the Rutherford Appleton Laboratory, Oxford, on 23–25 May 1983.

the interpretation of the colours of very distant galaxies, where the ultraviolet light is redshifted into visible wavelengths.

An alternative approach to seeking remote luminous galaxies is to perform deep counts of all galaxy images on a photographic plate. Ellis showed that counts obtained by several groups, including those at Durham with the AAT, were now probing cosmological distances. At 22nd magnitude in the blue, one expects to find many galaxies at a redshift of 0.5 or greater. Comparison of the available data suggests that evolution in blue luminosity may be required. The amount of evolution is rather modest, and in fact is very similar to that used by Bruzual in interpreting the colours of the high-redshift radio galaxies. This would again imply that many galaxies were bluer and more luminous in the past.

Where does all this leave us? The evidence for evolution in distant galaxies is growing. Unfortunately, it is impossible to arrive at a unique interpretation of the observations. There may be many possible evolutionary tracks that yield the mature galaxies we now see, and extrapolation into the past becomes exceedingly dangerous while we are still uncertain of what is happening in nearby galaxies and, indeed, in our own Galaxy. We require an improved understanding of the evolution of the youngest stars and of the oldest stars. We must discover how these processes depend on metallicity. We need to understand how star formation is initiated and to measure the initial distribution of stellar masses. And we cannot neglect the effects of galactic environment. Some recent UKIRT observations (N. Robertson, Imperial College, London) show that tidal interactions between galaxies may stimulate star formation. Other environmental effects that may plague distant galaxy observations include those of galactic cannibalism, whereby a luminous galaxy swallows its lesser neighbours, and galaxy mergers.

The goal of using distant galaxies to determine whether the Universe is open or closed seems as remote as ever, but our understanding of galactic evolution is likely to be improved considerably. The challenge posed by theories of galaxy formation is clear. Accumulating evidence from the large-scale distribution of galaxies (G. Efstathiou, Institute of Astronomy, Cambridge) points to the existence of large-scale coherence. Numerical simulations of galaxy clustering only match the observed correlation function if collapse on a large scale occurs at a relatively late epoch, corresponding to a redshift of less than three. Could galaxy formation coincide with this collapse phase, and have occurred at such a recent epoch? To prove or disprove this hypothesis is the immediate challenge facing the observers of remote galaxies. □

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Quantum mechanics

Phonokinetic analogue of the photoelectric effect?

from J.F. Allen

In this issue of *Nature* (p.325), Baird *et al.* describe how they have demonstrated the thermal energy equivalent to the photoelectric effect first described by Einstein to explain the observation that the kinetic energy of electrons ejected from metal surfaces by irradiation with light depends not on the intensity of the irradiant light but on its frequency. Einstein suggested that the phenomenon could be understood if the beam of light were treated as a stream of bombarding photons, with the light acting as quasi-particles of energy $h\nu$, where h was Planck's then newly proposed constant quantum of action and ν was the frequency. This was the first great achievement of quantum physics.

A little later, Millikan made confirmatory measurements of the photoelectric effect but at the same time suggested that Einstein's idea about discrete photons was pretty well discredited. Even Einstein later expressed doubts about quantum mechanics, and certainly 70 or 80 years ago it was intellectually difficult to treat an electromagnetic wave, such as light, as having at the same time both wave and particle properties.

Then 40 years ago Landau described the thermal excitation spectrum of superfluid helium in terms of phonons and rotons. He said that if light, as black-body radiation in a uniform temperature enclosure, could be treated as a kind of gas of photons with a defined energy density but with an unconserved number, so one could treat the random thermal motions of atoms in a liquid or solid as a gas of quasi-particle sound waves or phonons; again with total energy determined but with numbers of phonons unconserved. (The rotons need not concern us here.)

The treatment of thermal energy as a phonon gas has been very fruitful. Arising from it has been the suggestion, made from time to time, that there should be some kind of phonokinetic analogue of the photoelectric effect. Just as an electron coming out of a metal must have sufficient energy to overcome the work function, so a gas atom must overcome the binding energy, that is the latent heat per atom, in order to escape from the liquid surface.

Although saturated vapour pressures are usually quite high, the microscopic picture of evaporation is not very clear. What has been lacking has been the ability to produce an identifiable high-energy phonon to knock out an identifiable atom from the liquid.

This problem has now been solved by Baird, Hope and Wyatt in Exeter. In a bath of superfluid helium at 0.1K, where the

vapour pressure is effectively zero, they put a short powerful heat pulse into an immersed heater which produces a burst of phonons with a normal energy distribution. These are directed towards the bath surface and the ejected atoms impinge on a sensitive bolometer in the 'vacuum' vapour space above the liquid.

The phonon excitation spectrum of the superfluid is unusual. The low-energy phonons travel faster and reach the surface first, but they have a short mean free path and decay readily, giving a small bit of noise on the bolometer signal. They form a 'thermodynamic' background.

At higher energies, however, where the equivalent phonon temperature is of order 10K, the graph between ordinate energy and abscissa momentum is convex upwards, which means that normal decay, involving conservation of both energy and momentum, is no longer possible. Thus the high-energy phonons behave 'ballistically' with a mean free path of many centimetres, and their velocity and energy can be determined.

Baird *et al.* used a very simple and elegant device of a heater at a constant fixed distance below a bolometer, with the heater in the liquid and the bolometer in the space above it. The system could be raised or lowered to vary the liquid and vapour paths, keeping the total path constant. A collimator orifice was fixed just below the liquid surface, to ensure that the ballistic phonons of interest and the ejected atoms of interest travelled along the same straight line. It was, in effect, a time-of-flight spectrometer.

From the time taken for the phonons to go from heater to surface and for the ejected atom to go from the surface to bolometer, and from knowledge of the binding energy, it was found that the phonon energy equalled the sum of the binding energy plus the kinetic energy of the ejected atom.

The results were checked by adding a small number of ^3He atoms to the otherwise very pure ^4He , with the knowledge that ^3He atoms sit on a superfluid ^4He surface. The evaporated ^3He atoms, with their binding energy known, also had the correct kinetic energy.

Baird, Hope and Wyatt have thus clearly established that there is a one-to-one phonon-atom quantum evaporation, and that we can speak of a phonokinetic effect which is the analogue of the photoelectric effect. □

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Immunology

More sources of antibody diversity

from T.J. Kindt and A. Coutinho

In recent years, an explosion of information on DNA structure has drastically changed our perception of the multi-gene system that encodes antibodies. It is now recognized that antibody diversity arises from several sources, including: (1) a multiplicity of germ-line V -, D - and J -gene segments encoding each chain; (2) combinatorial assortment of the segments; (3) junctional diversity generated at the interfaces of the segments; (4) somatic mutation operating on the segments after assembly; and (5) combinatorial associations of heavy and light chains. There are also suggestions that gene conversion may contribute to expansion of antibody repertoires. Whereas formerly, the alternative between a germ-line or a somatic origin of antibody diversity was the heart of the debate, today's investigators attempt to assess the relative contribution of both germ-line and somatic mechanisms and to establish the differentiative stage at which they operate in the cells expressing immunoglobulin genes (B lymphocytes and their precursors).

Antibody diversity is most frequently studied in the responses to well defined antigens in inbred strains of mice, because of the homogeneity of the responses and the occurrence of serologically defined variable-region markers — recurrent idiotypes — transmitted as mendelian traits. The most representative of such idiomorph systems were discussed at a recent conference*.

Several investigators have attempted without success to use monoclonal anti-idiotypic antibodies or homologous and heterologous antisera to establish the structural correlates of idiotypes on families of related monoclonal antibodies with known amino-acid sequences. Putative assignments of an idiomorph to specific residues were invariably invalidated by analysis of additional antibodies. Nonetheless, idiomorph workers can provide some indication of the existence of a specific prototype variable region in a family of antibodies; and the data presented served as a springboard to consider both germ-line genes and somatic mechanisms giving rise to the antibodies defining each system.

It is now clear that there is a substantial repertoire of germ-line genes. The availability of probes for the V_H regions of several major idiomorph families allowed R. Riblet (Institute of Cancer Research, Philadelphia) to use Southern blot techniques to map the genes corresponding to

these families in several inbred and recombinant mouse strains. Although the polymorphism was not extensive, it was sufficient to allow distinction among different haplotypes.

Riblet's data indicate that genes belonging to a family (recognized by cross-hybridization with a given V_H probe) are inherited as a linked group of sequences with approximately 80 per cent homology. Five families have been characterized so far, and there are indications for another two or three. Each family contains around 10 genes, with a notable exception of one superfamily of about 40 genes. Comparisons of genes among families indicate that interfamilial homology is approximately 58–72 per cent for the coding sequences. Most cross-overs are found among families; there is an estimated 40 kilobases between each V_H family and a similar distance separates the D -region cluster from the nearest V family. The use of probes for five different families allowed determination of the following order of genes, beginning at the centromeric side of the IgH locus: *Hyt...J606 J558 S107 Q52 7183 D_H J_H C...PreAlb*.

The multiplicity of V_κ genes was also discussed (D. Gibson, University of Sherbrooke; M. Weigert, Institute for Cancer Research, Philadelphia) and although the evidence presented was not as direct as for the IgH locus, there was general agreement that the V_κ locus is organized in roughly 50 families with an average of six genes in each.

Comparison of germ-line gene segments to the sequences obtained for a panel of antibodies selected for idiomorph and/or antigen specificity allows assessment of the degree of somatic diversity operative in generation of antibodies within a given family. In some systems, for example in the case of anti-arsonate (D. Capra, University of Texas; M. Gefter, MIT), anti-phosphorylcholine and anti-oxazolone (O. Mäkelä, University of Helsinki; C. Milstein, MRC, Cambridge) antibodies, a single germ-line V_H gene nearly always associates with the same D and J_H segments to make up an antibody family. In other cases, such as the heterocytic anti-nitrophenyl response, at least three different V_H segments seem to be used in the dominant antibody family (K. Rajewski, University of Cologne; T. Imanishi-Kari, MIT; A. Bothwell, Yale University).

In the anti-carbohydrate responses — galactan (S. Rudikoff, NIH) and dextran (R. M. Perlmutter, Cal Tech) — multiple V_H and/or a large variety of D segments can be used. For the anti-carbohydrate anti-

bodies, junctional diversity appears particularly important to ensure conservation of the total length of the third complementarity-determining region (CDR3), which is encoded by the D segment. Thus, no matter the number of residues encoded by the D segment, the sites of recombination on both the 5' and 3' ends to the V and J segments, respectively, will vary to maintain that characteristic. The importance of junctional diversification and conservation of length of CDR3 was stressed in several other systems such as in anti-oxazolone antibodies (see M. Kaartinen *et al.* in this issue, p.320). The consistent finding of an Arg residue in CDR3 of anti-arsonate that must be generated by joining at the D - J boundary further underscores the importance of this mechanism. All these findings leave an open question of whether some precise V - D - J combinations represent products of a high probability for such recombinations or whether they merely reflect strong (antigen) selection.

It has been generally assumed that heavy-light chain pairing contributes to diversity, and this was indeed demonstrated for several systems where the same V_H or V_L gene segment, in different combinations, generated entirely different responses (see, for example, J. Rocca-Serra *et al.*, p.353).

The issue of somatic mutation was directly addressed by P. Gearhart (Carnegie Institute) who has evidence that somatic mutation operates only on rearranged genes in a manner consistent with nicking and repair of the nucleotide strands. Frequent (0.5 per cent) mutations were observed only in the environment of the rearranged V gene and were more likely to be found in clusters than random chance would dictate. Although it seems that somatic mutation occurs at some stage early in clonal development, the contention that it is concurrent with H-chain class switching was contested by data from several systems.

Formal proof of somatic mutation was offered by Weigert who studied the response to influenza virus. Murine monoclonal antibodies, all taken from the same mouse, were selected by their reactivity pattern on a panel of viruses. The antibodies so selected all expressed V_κ 21C subgroup (this subgroup is found on only 1 per cent of the normal IgG population). When seven hybridomas from the same mouse were analysed, it was evident that the V regions all varied in a stepwise fashion from a single ($V_{\kappa 21C}$) germ-line gene.

Also discussed was the subject of gene conversion. This mechanism is usually evoked when identical peptides or DNA segments (minigenes) are observed within several otherwise unrelated proteins or gene sequences. T. Rabbitts (MRC, Cambridge) discussed the existence of hot spots and the possible involvement of DNA Z regions in the regulation of conversion

*A conference on 'The structural correlates of idiomorph determinants' was held in Marseille on 24–27 April.

events. It remains difficult to assess the frequency and importance of gene conversion in the maintenance and expression of antibody genes.

It is now clear that there are several hundred V_H and V_L gene segments, approximately 12 D segments, 5 J_H and 4 J_L segments in the mouse germ line. The relative contribution of this inherited diversity and the later expansion of the repertoire by combinatorial and junctional diversification varies for each system. Although the full extent of antibody diversity that can be obtained from a given set of V -, D -, J -gene segments remains obscure, data on antibody and gene structure reveal considerable flexibility in manner of their assembly, since multiple combinations and permutations of the germ-line genes are possible. Whether there are favoured com-

binations and permutations or whether selection operates on an assortment of every possible configuration is still open to question. Furthermore, there is strong evidence that somatic mutation and/or gene conversion act on the rearranged V genes.

But two unrelated questions remain. When does mutation occur in the differentiation of lymphocytes from committed precursors with rearranged genes to antibody-secreting cells? What is the relative contribution of mutation to the available antibody repertoires? At present it seems likely that high rates of mutation are associated with antigen-driven clonal expansion. □

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Embryology

Lethal mutation in collagen gene

from Keith R. Willison

RUDOLPH Jaenisch's group recently reported the first success in artificially inducing a mutation that halts mammalian embryogenesis at a specific stage of development¹. Their technique, as discussed in *News and Views* at the time², is to infect early mouse embryos with murine leukaemia retrovirus, the DNA of which becomes stably inserted into the genome of some of the animals. Mutant strains can then be bred from animals carrying such insertions and one of these, Mov-13, carries a recessive embryonic lethal: homozygous embryos die at day 13 of gestation. In *Nature* this week, Schnieke *et al.*³ report that they have identified the site of the mutation as the gene encoding $\alpha 1(I)$ collagen. The exact mechanism of the lethal effect is not clear; but it may have interesting implications both for inherited disorders of collagen metabolism in man and for the future of retroviral mutagenesis as a tool for the exploration of mammalian development.

The discovery that the lethal mutation is in the gene encoding $\alpha 1(I)$ collagen was made possible by the availability of human and chicken cDNA clones that cross-hybridize with mouse DNA^{4,5}. The retroviral genome has integrated at the 5' end of the gene and blocks transcription. In normal embryos, abundant RNA transcripts from the $\alpha 1(I)$ collagen gene first become detectable at day 12 of gestation. In Mov-13 homozygous embryos, no stable $\alpha 1(I)$ transcripts are ever detectable and the embryos die from a general tissue necrosis that is first visible in the liver at day 12 of gestation.

The absence of malformations in the embryos suggests that type-1 collagen plays no part in morphogenesis up to this stage (12-day mouse embryos have prominent limb buds just beginning to digitate from

mesenchyme to precartilage for example). Since some type-1 collagen is serologically detectable in 9-day mouse embryos, Schnieke *et al.* suggest that inhibition of type-1 collagen synthesis may interfere with the laying-down of the extracellular matrix which is thought to be important in interactions between mesenchyme and epithelium. The failure of cells to differentiate in the absence of such interactions may ultimately be responsible for the death of the whole embryo.

The Mov-13 mouse strain should be directly relevant to the study of human disease because collagen defects are strongly implicated in some heritable disorders such as osteogenesis imperfecta. This group of disorders is characterized by brittle bones but is otherwise very heterogeneous. A case that resulted in perinatal death of the patient was recently described in this journal. The patient was heterozygous for an internal deletion of 0.5 kilobases in the pro- $\alpha 1(I)$ collagen gene which resulted in an in-frame mRNA transcript coding for a truncated polypeptide⁶. The type-1 procollagen trimers containing truncated pro- $\alpha 1(I)$ chains are rapidly degraded and this results in a decrease in the number available for fibre formation. Since Mov-13/+ mice are healthy however, it seems to be possible to cope with half as much $\alpha 1(I)$ collagen so long as it is normal (assuming no compensatory synthesis from the wild-type allele). Thus insertion mutagens that can produce null alleles may in fact be the most effective agents for producing viable recessive mutants.

What does this result mean for mammalian developmental geneticists? It has recently been argued in *Nature*⁷ that mammalian developmental genetics cannot hope to compete with research on *Drosophila* whose morphogenetic

mutants, to say nothing of its more manageable genome size, have lately been so elegantly exploited and for which a transformation system analogous to Jaenisch's retroviral mutagenesis has now been developed⁸.

In fact it is germane to the consideration of the collagen lethal mutation to discuss some estimates from *Drosophila melanogaster* of the number of genes required for any developmental process. Garcia-Bellido and Robbins⁹ have studied the viability of female germ-line cells homozygous for zygotic lethals in *D. melanogaster*. Using mitotic recombination they show that the germ line is more sensitive to mutations than clones of epidermal cells: more than twice as many of the zygotic lethals are lethal in the germ line than the epidermis. As 9 per cent of zygotic lethals are lethal in epidermal clones¹⁰ it is probable that more than 20 per cent of cell-autonomous genes are required for female germ-cell development. However, there are clearly many genes whose products are not and this class would include non-cell-autonomous genes coding for extracellular matrix components.

Mov-13 was generated by infecting post-implantation embryos with virus and was the only insertion discovered in several hundred offspring, insertion being assayed by viraemia. Schnieke *et al.* speculate that the murine leukaemia virus provirus preferentially inserts into actively transcribed DNA, a testable hypothesis since it should be possible to show whether primordial germ cells synthesize $\alpha 1(I)$ collagen. If this is the case, it reduces the target size since only 3 per cent of the genome is transcribed. Because collagen is a secreted product, the effects of mutations in the gene encoding it would be expected to be non-cell-autonomous. These considerations may thus explain why the collagen gene should have accepted an integrating provirus, and why it was possible to isolate the resulting mutant. It would have been all the more exciting to have discovered a new gene by Jaenisch's technique rather than a previously cloned, somatically transcribed one. However, we now know that a fairly early-acting recessive lethal mutation in mouse embryogenesis maps in the $\alpha 1(I)$ collagen gene and this is an important result for the field of developmental genetics. □

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Plant biochemistry

Mobile genes of chloroplasts and the promiscuity of DNA

from John Ellis

A KEY problem in evolutionary biology is to account for the origins and properties of the genetic systems that occur outside the nucleus in all eukaryotic cells. It seems a high cost to the plant cell to specify at least 200 extra polypeptides involved in replication, transcription and translation by mitochondria and chloroplasts in order to make a rather small number of polypeptides inside these organelles instead of outside them. In the case of chloroplasts, the explanation may lie in their proposed origin in free-living photosynthetic cyanobacteria through endosymbiosis^{1,2}.

Extant cyanobacteria which carry out oxygenic photosynthesis contain the CO₂-fixing enzyme ribulose biphosphate carboxylase/oxygenase, which consists of catalytic large subunits and regulatory small subunits. In these cyanobacteria, both types of subunit are encoded in the same genome along with all the other proteins necessary for a phototrophic prokaryote.

A similar enzyme is found inside the chloroplasts of photosynthetic eukaryotes, but in these organisms the two types of subunit are encoded and synthesized within different genetic compartments³. The large subunit is a product of the chloroplast genome, while the small subunit is synthesized as a higher-molecular-weight precursor by cytoplasmic ribosomes translating a nuclear-encoded mRNA. Post-translational transport of this precursor into the chloroplast is accompanied by cleavage to the mature size by a soluble chloroplast protease which removes the amino-terminal extension⁴. The small subunit is representative of the majority of chloroplast polypeptides, since these are also encoded in nuclear genes and synthesized as precursors by cytoplasmic ribosomes⁵.

Thus if the endosymbiont hypothesis is correct, a massive transfer of genes from symbiont to nucleus must have occurred during evolution; and one might expect that intermediate stages may have persisted. In this issue of *Nature*⁶, Heinhorst and Shively report that in the organism *Cyanophora paradoxa*, the carboxylase

small subunit is encoded not in the nuclear genome but in the genome of the photosynthetic organelle.

The protist *C. paradoxa* contains two to four intracellular structures termed cyanelles, each surrounded by a vacuolar membrane; the cyanelles contain unstacked thylakoids and photosynthetic pigments typical of free-living cyanobacteria, and are invested by a thin peptidoglycan cell wall⁷. Cyanelles were first regarded as ingested cyanobacteria, but their genome resembles that of chloroplasts both in structure (a circular molecule containing an inverted repeat encoding rRNA) and in size (molecular weight about 10⁶). This genome is too small to encode enough proteins for a free-living organism⁸. Should cyanelles then be regarded as chloroplasts? But in this event, it would be expected that the carboxylase small subunit present in cyanelles would be encoded in the nuclear genome of the protist, and Heinhorst and Shively⁶ have shown that this is not the case. Use of a cloned cDNA hybridization probe prepared from the small-subunit mRNA of *Pisum sativum* demonstrates that the gene for the small subunit in *Cyanophora* resides near that for the large subunit in the cyanelle genome. (The hybridization was performed at low stringency to allow for the variability of the small subunit and the evolutionary distance between *Cyanophora* and *Pisum*.) Similar results were reported for *Cyanophora* by H.J. Bohnert, E.J. Crouse, W. Löffelhardt and H. Mücke at a recent meeting in Tübingen*.

These observations support the view that cyanelles 'represent a bridge between cyanobacteria and chloroplasts'⁹, and suggest that the location of the small-subunit gene should be determined in a wider range of eukaryotes, especially among the more primitive algae where transfer of the gene to the nucleus may not have occurred. Evidence was presented at the Tübingen

colloquium by K. Zetsche, K. Steinmüller and M. Kaling that in some red algae, the carboxylase small-subunit mRNA is present in the same poly(A)⁻ RNA fraction that contains the large-subunit mRNA; in other eukaryotes, such as *Pisum*, the small-subunit mRNA contains poly(A) while the large-subunit mRNA does not. This suggests that in these red algae the small subunit is synthesized inside the chloroplast, although direct evidence for this conclusion was not presented. Of special interest is the finding that the small subunit synthesized by incubating red algal poly(A)⁻ RNA with a reticulocyte lysate is made not as a precursor but at its mature size. This observation raises the question as to the evolutionary origin of the amino-terminal extension characteristic of cytoplasmically synthesized chloroplast proteins.

It is striking that in the four species so far examined (soybean, pea, wheat and *Chlamydomonas*), the amino terminus of the mature small subunit is occupied by methionine, even though this is not the first amino acid to be polymerized¹⁰. The presence of methionine at this position may be a coincidence, but a more interesting possibility is that it reflects the evolutionary origin of the precursor. Fusion of a prokaryotic gene for mature-sized small subunit possessing methionine as its initiating amino acid with a DNA sequence specifying transport across the chloroplast envelope would produce the observed amino acid sequence. The origin of the transport DNA sequence is a mystery.

The unusual location of the carboxylase small-subunit gene in *Cyanophora* supports the inference of the endosymbiont hypothesis that DNA leaves the symbiont as it evolves into an organelle. Presumably duplicate copies of the exported sequences are retained within the organelle until the nuclear sequences become fully functional by addition of the information for the amino-terminal extension of the polypeptides. Is there evidence for shared nucleotide sequences between chloroplast and nucleus in extant species?

In an earlier article in *News and Views*, I proposed the term 'promiscuous DNA' to describe nucleotide sequences found in more than one of the three membrane-bound organellar genetic compartments of eukaryotes¹¹. Examples of promiscuous DNA are mitochondrial sequences present in the nucleus of yeast¹² and chloroplast sequences present in the mitochondria of maize¹³. A further example is reported by Timmis and Scott¹⁴ who find that the nuclear DNA of spinach contains in-

*The Second International Colloquium on Endocytobiology was held in Tübingen on 11-15 April 1983.

ERRATA

In the *News and Views* article 'New light on climate from old isotope ratios' by Pieter M. Grootes (*Nature* 303, 753-754; 1983), there was a mistake in the third paragraph. The first sentence of that paragraph should read:

"The d term, introduced by Dansgaard², is the constant in a simple empirical relationship between the hydrogen and oxygen isotopic composition of worldwide freshwater sources ($d = \delta D - 8\delta^{18}O$ where δ is the relative difference in

isotopic composition between the sample and a standard, expressed in units per mill)."

In the article by David Blow in last week's *News and Views* on 'Computer cues to combat hypertension' (*Nature* 304, 213-214; 1983), the name of the third author of the paper to which the *News and Views* article referred and which appeared in the same issue (p.273-275) was erroneously missed out. Thus Laurence Pearl should have been acknowledged in the first paragraph and in reference 1.

egrated sequences homologous with most of the chloroplast genome. These results were obtained by hybridizing cloned chloroplast DNA sequences and restriction fragments of DNA isolated from spinach root nuclei. The hybridization patterns are difficult to explain by contamination of nuclear DNA by either mitochondrial or chloroplast DNA, but more definitive data will come only from sequence studies on nuclear DNA fragments which have been cloned.

The chloroplast DNA sequences that hybridize to spinach nuclear DNA include a fragment confined to the gene for the carboxylase large subunit. This nuclear sequence for the large subunit is not functional so far as is known, but this observation does raise the possibility that in some as yet unstudied species, this polypeptide is not a product of the chloroplast genome but is encoded by a nuclear gene. Such a species would be worth identifying because the carboxylase activity of the large subunit is the primary biochemical determinant of plant productivity. Attempts to improve the inefficient catalytic activity of the carboxylase by genetic engineering techniques are hampered both

by our inability to introduce DNA into the chloroplast and by the necessity to ensure that the introduced gene is in all the chloroplasts of the engineered plant cell. All the transformation systems being devised for plants are based on insertion of genes into the nuclear genome, so the identification of species that encode this vitally important subunit in the nucleus could turn out to be the most significant result of the academic interest in the evolutionary origin of chloroplasts. □

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RNA processing

Sequences that signal where to splice

from S.M. Mount

THE discovery of a short sequence critical to the removal of intervening sequences (introns) from the transcripts of nuclear genes which code for protein in the yeast *Saccharomyces cerevisiae* was recently reported in *Cell*¹. This finding promises to shed light on RNA processing in all eukaryotic species.

Until recently, there was every expectation that messenger RNA splicing in yeast would be little different from the same process in higher eukaryotes. All five yeast introns sequenced so far²⁻⁷ have at their termini (the splice sites) sequences which conform to the well conserved consensus sequences derived from splice sites of higher eukaryotes^{8,9}. Especially important is their agreement with 'Chambon's rule', which holds that G-T will be the first two nucleotides of any intron, and A-G will be the last¹⁰. However, there is an early report that a mouse β -globin gene was not properly spliced in yeast¹¹, and Langford *et al.*¹² have recently shown that yeast will not remove duck or amoeba introns, even when a yeast intron is properly excised from the same transcript. Thus, yeast splicing must require a signal of some sort which is not present in genes from other species.

The identification of such a signal was reported at a recent meeting* by two

groups: C. Langford and D. Gallwitz at the University of Marburg and a group at Brandeis University headed by M. Rosbash. Langford and Gallwitz have been studying removal of the one intron present in yeast actin, and the Brandeis group has been looking at removal of a ribosomal protein (rp51) intron from the transcripts of a rp51- β -galactosidase hybrid gene. Both have found that sequences between 35 and 90 nucleotides upstream of the 3' end of the intron are required for the production of mature mRNA *in vivo*. This region of both introns contains a heptanucleotide, TACTAAC, which, interestingly, occurs between 20 and 55 nucleotides upstream of the 3' splice site in all known yeast intron sequences.

Langford and Gallwitz have further discovered a remarkable capacity of this region to dictate 3' splice-site choice. They constructed a number of variant actin genes in which the TACTAAC sequence was retained but the adjacent 3' splice site was removed. All such constructs (four are described in the *Cell* paper¹ and a fifth was reported at the meeting) yield processed RNA. The 3' splice sites always occur at an A-G dinucleotide just downstream (between 15 and 67 nucleotides) of the TACTAAC sequence. Furthermore, the A-G is generally, but not always, the first A-G 3' of the TACTAAC sequence.

These results suggest that some component of the splicing complex first recognizes UACUAAC (in the RNA) and then scans the intron in a 5' to 3' direction until it finds the next A-G dinucleotide. Splicing does not occur at the first A-G in two cases where this A-G lies within 10 nucleotides of UACUAAC, perhaps because the search does not begin precisely at the end of the UACUAAC, but a little downstream. In two examples splicing is not restricted to a single A-G dinucleotide. One of these two cases involves an A-G which may border on being too close to TACTAAC (15 nucleotides), and the other involves splicing at AAG, which agrees with the GT...AG rule, but not with the expanded (C/T)AG consensus to which all natural yeast 3' splice sites conform.

One result, reported by N. Kaufer and J. Warner (Albert Einstein College of Medicine) called the universality of the TACTAAC sequence into question. In an analysis of the yeast gene *CYH2*, they found splicing of some sort, still uncharacterized as to the location of the splice sites involved, in a construct from which a restriction fragment including the 3' splice site and the TACTAAC sequence has been deleted. Identification of the 3' splice site used in this case should immediately reveal whether there is a TACTAAC-like sequence involved.

What features of this phenomenon in yeast can be applied to mRNA splicing in higher eukaryotes? The sequence TACTAAC is not present in more than a few introns in higher eukaryotes, and clearly cannot be responsible for 3' splice-site choice in all species. Furthermore, deletions of material from the corresponding region of introns in the genes of higher eukaryotes does not have any effect on splicing, which precludes any mechanism as specific as that of yeast. There is, however, a pyrimidine-rich stretch directly preceding all 3' splice sites: the region 5-15 nucleotides upstream of a 3' splice site is, on average, 79 per cent pyrimidines, and does not contain the dinucleotide A-G⁸. This region may substitute for the TACTAAC sequence. Langford's and Gallwitz's results show that the pyrimidine-rich region is not required for splicing in yeast; but B. Wieringa (working with C. Weissmann at the Institute for Molecular Biology in Zurich) has shown that it must be intact for the normal recognition of the 3' splice site of the rabbit β -globin intron. When the pyrimidines upstream of this 3' splice site are replaced by an *Xba* linker (CTCTAGAG) attached to sequences normally occurring further upstream in the same intron, splicing at this site is abolished. Normal splicing is preserved if 15 nucleotides of the natural 3' splice site are left intact, but not when only 12 remain. Thus, the pyrimidine-rich stretch between 5 and 15 nucleotides upstream of the 3' splice sites of higher eukaryotes is a functional analogue of TACTAAC in the limited sense that both

*The second Annual Meeting on RNA processing was held at Cold Spring Harbor on 18-22 May 1983.

are required for splicing at a downstream A-G. It will be interesting to see whether this analogy will extend further. One difficulty is that A-G has been seen at positions -5, -4 in two vertebrate introns^{13,14}.

A second possibility, advocated by Rosbash's laboratory, is that UACUAAC, present in the mRNA precursor, plays a part in 5' splice-site recognition. They have observed that UACUAAC is itself a site of cleavage. In trying to understand this result they noticed that the 5' end of the U1 RNA, which is proposed to recognize 5' splice sites¹⁵⁻¹⁷, is homologous to UACUAAC (the U1 sequence is UACUUAAC). Thus, they reasoned that UACUAAC might interact with the 5' splice site, the cleavage of UACUAAC resulting in some way from this interaction. This proposal is supported by their observation that UACUAAC cleavage is inhibited in a 5' splice-site mutant.

Whichever possibility is upheld by later results, research on splicing in yeast should elaborate a mechanism of splice-site

recognition that could apply, if only in altered form, to higher organisms. □

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Plant ecology

Photosynthetic pathways in aquatic plants

from Peter D. Moore

THE C_4 and crassulacean acid metabolism (CAM) photosynthetic systems are generally associated with terrestrial plants growing in habitats where water stress and high temperature are frequently encountered¹⁻³. The efficiency of carbon accumulation resulting from the elimination of photorespiration permits these plants the luxury of closer stomatal control leading to lower water losses. In the case of CAM plants, stomata open for gaseous exchange only at night when potential water losses are minimal.

Some C_4 species are known from marine saline habitats such as estuaries and salt marshes, for example *Spartina anglica*⁴, but it is surprising to find that some of the biochemical features associated with this photosynthetic strategy have been described recently in submerged freshwater aquatic plants. One such plant is the Canadian pondweed, *Elodea canadensis*⁵. When *Elodea* was supplied with $^{14}CO_2$, 45 per cent of the label ended up in C_4 acids rather than C_3 compounds.

One possible explanation of the occurrence of such a pathway in an aquatic plant is the diurnal fluctuation in dissolved carbon dioxide in a freshwater body, especially if it contains a high density of aquatic plants. Carbon dioxide concentration would tend to be high during the night and low during the day, when it would be in demand for photosynthetic fixation. A mechanism whereby night fixation of CO_2 could take place (as in CAM plants) could therefore be of considerable selective

value. But although a C_4 mechanism is present, there is no evidence of nocturnal net carbon uptake occurring in *Elodea*.

Work on other aquatic plants, however, has shown that at least some species can enhance their carbon metabolism by nighttime fixation. For example, the North American submerged water plant *Scirpus subterminalis* has been shown to accumulate malate during the dark (at least in part derived from respiratory CO_2) which accounted for 12 per cent of the net photosynthesis⁶.

Whilst working with another aquatic macrophyte, *Hydrilla verticillata*, Holaday and Bowes⁷ found a considerable variation in CO_2 compensation point (the CO_2 concentration below which a plant takes up less CO_2 by photosynthesis than it gives out by respiration), which depended on growth conditions. Winter collections had high compensation points and summer collections low ones. The plants with low compensation points, when provided with $^{14}CO_2$, showed a 60 per cent incorporation of labelled carbon into the C_4 acids malate and aspartate, and a high activity of phosphoenolpyruvate carboxylase, the first enzyme in the C_4 fixation pathway. Clearly these plants have the ability to vary the CO_2 compensation point in response to environmental demands by the facultative assumption of a C_4 fixation system.

Just how widespread C_4 fixation is among water plants is not yet known, but Keeley and Morton⁸ have recently conducted a survey of 30 species of aquatic

plants found in a variety of pools and lakes, ranging from oligotrophic to eutrophic, and have found only two species which showed overnight accumulation of acids. These are *Crassula aquatica*, a plant of the margins of temporary ponds, and the shoreweed, *Littorella uniflora*, found mainly along the gravelly edges of nutrient-poor lakes with fluctuating water levels. The authors interpreted their results as support for the hypothesis that aquatic plants can increase their efficiency of carbon utilization in this way under conditions where carbon availability is likely to be growth limiting.

From a taxonomic and an evolutionary point of view, one of the most interesting groups in which such diurnal acid metabolism has emerged is the fern *Isoetes*. The mechanism seems to be very uncommon among pteridophytes, having previously been reported only in two tropical ferns⁹, and it was first reported in *Isoetes* by Keeley¹⁰. He found an overnight accumulation of malic acid in the leaves of *Isoetes howellii*, which grows in seasonal pools produced by the winter and spring rains of California, which dry out during the summer drought. Keeley has since surveyed 11 other *Isoetes* species¹¹ (the genus contains about 70 species in all) derived from locations as far apart as Guatemala, British Columbia and North Wales. All the species were found to exhibit diurnal acid metabolism, and Keeley has proposed⁸ that it may be present in all members of the genus. Many of the species, like *I. howellii*, become emersed during dry periods and the diurnal variations in leaf acid content then become less marked.

These studies are of interest and importance both in their further demonstration of the taxonomically widespread nature of C_4 and CAM mechanisms of carbon fixation and also in that they indicate another ecological advantage of such a mechanism in an unexpected habitat. In this case, a daytime shortage of dissolved inorganic carbon for use in photosynthesis in aquatic ecosystems has led to the evolutionary selection of a biochemical pathway which has previously been associated largely with arid environments where its value relates to economy in water loss rather than a general scarcity of carbon. □

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Astrophysical consequences of a violation of the strong equivalence principle

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A previous analysis of the consequences of a violation of the strong equivalence principle (SEP) for massive particles has now been extended to the case of photons and leads to the prediction that the photon number is conserved. Comparison of the predictions of our theory with observations, especially those on big-bang nucleosynthesis, shows that a violation of the SEP is not ruled out by the data available, and that further direct measurements are required.

THE strong equivalence principle (SEP) states that the outcome of any gravitational or non-gravitational experiment is independent of where and when in the Universe it is performed¹, or, in particular, that in spite of changes in the global distribution of matter in the Universe, no influence is to be felt by local gravitational and non-gravitational experiments performed at different epochs. It can be shown¹ that the SEP requires that all clocks in nature be equivalent, or that the ratio of their intrinsic units of time be constant. An SEP violation occurs when the ratio of the period of an atomic (or nuclear or weak) clock to that of a gravitational clock (such as a planet revolving about a star) is not constant in time. If by Δt_E we denote a time interval recorded with a gravitational clock and by Δt that recorded by an atomic clock, we characterize an SEP violation by a non-null value of the time derivative $\dot{\beta}_a$ of the quantity β_a defined by $\Delta t_E = \beta_a \Delta t$ (refs 1, 2).

The question whether or not the SEP is violated can be resolved directly by observations. For example, by using an atomic clock to record the flight time of photons to and from Mars, one can monitor the period of Mars orbiting the Sun, which is a gravitational clock. The analysis of the radar ranging data would then lead to a determination of $\dot{\beta}_a$ (ref. 3).

To stimulate an observational search to test the SEP, it is necessary to show that an SEP violation is at least not ruled out by data already existing. To carry out the analysis, one needs a theoretical scheme and in particular to know whether an SEP violation would affect both gravitational and non-gravitational physics, as is possible in principle. Before our work, it was generally assumed that an SEP violation would affect only gravitational phenomena and that all non-gravitational physics is independent of it. To be specific, it was assumed that both one- and many-particles relations for fermions and bosons remain independent of β_a even when $\dot{\beta}_a \neq 0$. The prototype of such theories is that of Brans and Dicke.

We have recently constructed an SEP violating framework¹ that does not require that an SEP-violation should affect only gravitational phenomena.

Two main results have emerged. (1) Where massive particles are concerned, while one-particle relations remain unchanged, many-body relations (for example, the relation between pressure P and density ρ) are affected by β_a , in contrast with the assumptions of previous theories where this did not occur. (2) In order for atomic and gravitational clocks to run at different rates, the relation between the gravitational constant G and the function β_a must be of the form $G \sim \beta_a^{-g}$, with $g = 2$.

Our previous work¹ did not deal with photons, but we have now been able to extend it so as to arrive at a framework for photons which is valid for any g . In so doing, we discover that our previous photon theory⁴ holds only for $g = 1$. That theory is therefore no longer tenable because it is inconsistent with the condition (2) above that for the SEP to be violated, g must

be 2. We stress that the $g = 1$ theory must be abandoned not for observational reasons but on the grounds of theoretical consistency. (The implications of the two photon theories for the 3 K radiation are compared in what follows.)

The new photon theory allows us to construct five new tests of SEP violation. Together with previous tests of a cosmological nature^{5,6}, we conclude that an SEP violation has been found to be compatible with a wide range of data from astrophysics to geophysics. The results do not prove that an SEP violation must occur, but they constitute the assurance required for an observational search to be undertaken³. (As in ref. 1, β denotes general units; in atomic units, AU, $\beta = \beta_a$; in gravitational units, EU, $\beta = 1$).

Photons

Single particle relations: We begin with the derivation of the equation for the propagation of photons using the equation of motion in terms of the four velocity u^α of a test particle¹ of mass μ

$$u^\alpha_{;\nu} u^\nu + \frac{(\mu \beta^{2-g})_{;\nu}}{(\mu \beta^{2-g})} \Delta^\nu = 0 \quad (1)$$

The second term in equation (1), involving the 'projection operator' $\Delta_{\alpha\nu} = u_\alpha u_\nu - g_{\alpha\nu}$ ($\Delta_{\alpha\nu} u^\alpha u^\nu = 0$) is a deviation from the standard geodesic equation $u^\alpha_{;\nu} u^\nu = 0$, (where the semi-colon denotes the covariant derivative) arising from the possible non-constancy of μ , β and G .

Introducing the momentum $p_\alpha = \mu u_\alpha$ (with $c = 1$), where $p^\alpha p_\alpha = \mu^2$, equation (1) can be rewritten as

$$(\beta^{2-g} p_\alpha)_{;\nu} p^\nu - \frac{1}{2} \beta^{g-2} (\beta^{4-2g} p^\nu p_\nu)_{;\alpha} = 0 \quad (2)$$

For zero rest mass particles, $p^\nu p_\nu = 0$, this reduces to

$$(\beta^{2-g} p_\alpha)_{;\nu} p^\nu = 0 \quad (3)$$

which is equivalent to equation (3.10) of ref. 4 if one chooses $g = 1$.

To derive the energy-frequency (ϵ_γ , ν) relation, note that the energy of a photon of momentum p^α , as measured by an observer with velocity u^α , is $\epsilon_\gamma = u^\alpha p_\alpha$. As in the standard case, using a Robertson-Walker (RW) metric, equation (3) implies, for co-moving observers $u^\alpha = \delta^\alpha_0$,

$$p_0 \sim \frac{\beta^{g-2}}{R} \quad \text{and} \quad \epsilon_\gamma = \delta^\alpha_0 p_\alpha = \frac{h\nu}{\beta^{2-g}} \quad (4)$$

where h is a constant and where we have used the standard redshift relation $\nu R = \text{constant}$, as required by the fact that the photon path is null. The energy-frequency relation (4) can be shown to hold true for a general metric. It can be seen that if $g = 1$, or $G \sim \beta^{-1}$, the relationship $\epsilon_\gamma = h\nu/\beta$ derived in ref. 4 (equation (3.15)) is recovered.

Since we have shown¹ that g must be 2, we conclude that the two relations in equation (4) are identical with those of standard theory.

Photons in a beam: Let us now consider a beam of photons described by an energy momentum tensor of the standard form $T_{\alpha\nu} = f u_\alpha u_\nu$, where the function f will be determined later. In our framework, the possible non-constancy of β and G changes the standard conservation law $T^{\alpha\nu}_{;\nu} = 0$ to (equation (4.1) of ref. 4)

$$T^{\alpha\nu}_{;\nu} + (2-g)\frac{\beta_{,\nu}}{\beta}T^{\alpha\nu} - \frac{\beta_{,\lambda}}{\beta}T^\lambda_\alpha = 0 \quad (5)$$

With the help of equation (3), we obtain

$$(fp^\nu)_{;\nu} = 0 \quad (6)$$

To determine the function f , we note that the photon energy density, $\rho_\gamma \equiv n_\gamma \epsilon_\gamma$ (where n_γ is the photon number density) measured by an observer with velocity u^α , is given by $\rho_\gamma = T^{\alpha\nu} u_\alpha u_\nu = f(p^\alpha u_\alpha)^2 = f\epsilon_\gamma^2$. Therefore, $f = n_\gamma/\epsilon_\gamma$. For observers momentarily at rest, so that $u^\alpha = g_{00}^{-1/2}\delta^\alpha_0$ and $\epsilon_\gamma = p^{00}_{g_{00}}^{1/2}$ integration of equation (6) yields

$$\int \sqrt{\hat{g}} n_\gamma g_{00}^{-1/2} dx^3 \equiv N_\gamma = \text{constant} \quad (7)$$

where $\hat{g} = |\det g_{\mu\nu}|^{1/2}$. The number of photons N_γ is therefore conserved independently of the value of g .

Furthermore, we find that the cross-section A of the beam satisfies the relation $A_{;\alpha}\beta^{2-g}p^\alpha = A(\beta^{2-g}p^\alpha)_{;\alpha}$, which using equations (4) and (6), yields $n_\gamma A/\nu = \text{constant}$. As in standard theory, this expresses the conservation of the number of photons passing through different cross-sections along the beam.

Adiabatic photon gas: Consider black-body radiation in a box of volume V . Integrating equation (5) with $p = n_\gamma kT = 1/3\rho_\gamma$, one obtains

$$\rho_\gamma \sim \beta^{g-2} V^{-4/3}, \quad N_\gamma TV^{1/3} \beta^{2-g} = \text{constant} \quad (8)$$

The photon number N_γ is defined as $n_\gamma V = \rho_\gamma V/\epsilon_\gamma$. Since the frequency ν of the standing photon waves is related to V by $\nu \sim V^{-1/3}$, it follows that

$$N_\gamma = \text{constant} \quad (9)$$

independently of g . Therefore, the number of black-body photons in a box is constant independently of whether the SEP is violated or not. We note that the relation $N_\gamma \sim \beta_a^{g-1}$ used in ref. 4 is consistent with equation (9) only if $g = 1$. From equation (8) with the help of equation (9) it follows that

$$\rho_\gamma \sim \beta^{3(2-g)} N_\gamma^4 T^4 \sim \beta^{3(2-g)} T^4 \quad (10)$$

which reduces to the standard Stefan-Boltzmann relation for $g = 2$.

Black-body radiation spectrum: To obtain the differential spectral density $\rho_{\gamma\nu}$ (with $\rho_{\gamma\nu} d\epsilon = \rho_\gamma d\nu$ in an obvious notation) we use equation (8) in the form $\beta^{2-g} V^{4/3} \rho_\gamma = \text{constant}$ whence (with N_γ constant)

$$\begin{aligned} \rho_{\gamma\nu} &\sim \beta^{3(2-g)} \epsilon_\gamma^3 f(\epsilon_\gamma/T) \\ \rho_{\gamma\nu} &\sim \beta^{g-2} \nu^3 f(\nu/\beta^{2-g}T) \end{aligned} \quad (11)$$

where $f(x)$ is a universal function of x . Putting $g = 1$, the second of equations (11) reduces to equation (5.17) of ref. 4. With $g = 2$, the spectra are identical with standard spectra.

Photons and massive particles: Our conclusion (equation (9)) that the photon number is constant must be contrasted with our earlier result¹ for the particle number N_p

$$N_p \sim \frac{1}{\beta_a G} \sim \beta_a \quad (g = 2) \quad (12)$$

what this implies is that while all photon relations are unaffected by the presence of an SEP violation, massive particle numbers are affected. To take the comparison further, consider a system of massive particles described by $T_{\alpha\nu} = (p + \rho)u_\alpha u_\nu - p g_{\alpha\nu}$, where

$\rho = \rho_0 + \rho_*$ and $\rho_0 = mn$ where $n = N_p/V$ and $p = \Gamma\rho_* = nkT$. Integrating equation (5), and using the expressions for ρ_0 in equation (4.23) of ref. 4 or equation (2.47) of ref. 7, we conclude that

$$\rho_* V^\gamma \beta^\Omega = \text{constant} \quad \text{or} \quad N_p TV^{\gamma-1} \beta^\Omega = \text{constant} \quad (13)$$

where $\Omega = 3\Gamma + 1 - g$, $\gamma = 1 + \Gamma$. Eliminating T , one further gets (in AU)

$$p \sim \rho_0^\gamma N_p^{-\gamma} \beta_a^{-\Omega} \quad \text{or} \quad p \sim \rho_0^\gamma \beta_a^{-(\gamma-1)(2+g)} \quad (14)$$

showing that the p - ρ_0 relation is indeed altered by possible violation of the SEP. Equation (14) was used in ref. 8 to study the problem of the Earth's palaeoradius.

The results of the proceeding calculations may be summarized as follows:

- (1) The photon number N_γ and the particle number N_p behave differently as a consequence of an SEP violation. While N_γ is constant as in equations (7) and (9), N_p is not, equation (12), since g must equal 2 if gravitational and atomic clocks¹ are not to be equivalent.
- (2) For $g = 2$, all photon relations are unaffected by SEP violation.
- (3) Accordingly, SEP violation cannot be detected by the study of free photons, because it affects only the behaviour of massive particles.

Cosmology

In our previous work (see, for example, ref. 5) we have compared the predictions of our earlier calculations with various cosmological data, especially those pertaining to the variation of magnitude or angular diameter with redshift for galaxies (including radiogalaxies) and for QSOs. (The results of refs 5, 6 apply, provided that $g = 2$.) These comparisons show that an SEP violation is not inconsistent with the data provided that $G \sim t^{-1}$ or, equivalently, that $\beta_a \sim t^{1/2}$. We now proceed to a consideration of the implications of our new results.

Age of stars and globular clusters

If G were larger in the past, stellar luminosities would have been great. For the Sun, detailed numerical computations are available in the literature⁹⁻¹³ for the two cases: $M \sim t^0$ and $M \sim t^2$, where M is the total mass both using $G \sim t^{-1}$. In the first case, the radioactive age of the Sun can be matched if the hydrogen and metal abundances X and Z , respectively are $X = 0.82$ and $Z = 0.017$, whereas for $M \sim t^2$, the required abundances are $X = 0.725 - 0.73$ and $Z = 0.02$. The first set is considered unacceptable, since present estimates of the helium abundance Y are $0.20 \leq Y \leq 0.30$, whereas the second set is clearly acceptable. In the present framework, because of $G\beta_a^2 = \text{constant}$ and $\beta_a GM = \text{constant}$ (ref. 7), we have $M(t) \sim G^{-1/2}(t) \sim t^{1/2}$, a case intermediate between those investigated numerically so far. The final result must therefore be $0.73 < X < 0.82$, leading to an acceptable helium abundance Y .

For globular clusters, unlike the case of the Sun, the HR diagram provides a well defined turn-off position, and so since a larger luminosity in the past implies a shorter lifetime, one may avoid the difficulties created by an increase in the Hubble constant to $80-100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, as recently suggested. In fact, such large values would imply a maximum age of the Universe of $10-12 \times 10^9 \text{ yr}$, lower than the age of some globular clusters computed from standard theory, the estimated age of $15 \times 10^9 \text{ yr}$ for M15, for example.

Past temperature of Earth

A larger G in the past implies a higher solar luminosity L and hence a higher effective temperature, T , on the surface of the Earth. Quantitative estimates of the effect, first done by Teller¹⁴ lead to

$$L \sim G^8 \quad T \sim G^{2.5} \quad (15)$$

This implies that $4 \times 10^9 \text{ yr}$ ago, $T = 500 \text{ K}$, well above the boiling point of seawater and in apparent contradiction with

well substantiated data that liquid water existed on the Earth as long as 3.8×10^9 yr ago. We have used the power law $G/G_0 = t_0/t$, $t_0 = 15 \times 10^9$ yr as well as the present temperature $T_0 = 250$ K, corresponding to no greenhouse effect. Also included in the estimate of $T = 500$ K is the prediction by standard stellar evolution¹⁵ that 4×10^9 yr ago the Sun's luminosity was 30–40% lower than today, implying an 8% reduction in T .

Within the present framework, the equations for the hydrostatic equilibrium equation, the radiative transfer and stellar luminosity ($L = \epsilon M$, where $\epsilon \sim \rho T^n$ is the nuclear energy rate^{9–13}) and the equations of state $pV = N_p kT$, $\rho_p \sim T^4$, together with $G = G(t)$ and $M \sim G^{-1/2} \sim \beta_a$ yield by homology arguments

$$L \sim G^\alpha M^\delta \sim G^{s/2} \sim \beta_a^{-s}$$

where

$$s = 2\alpha - \delta = \frac{59 + 18n}{5 + 2n} \quad (16)$$

With $n = 4$ for the Sun, and remembering that the Earth–Sun distance D scales like $D_0 = \beta_a D$, we derive

$$L \sim G^5 \quad T \sim G \quad (17)$$

which implies that 4×10^9 yr ago, $T \approx 313$ K, well below the boiling point of seawater.

Although a fully reliable result must await an atmospheric calculation as well as the evaluation of the L – G relation based on a detailed stellar evolutionary model, the estimates presented here indicate the contrast with the results obtained from previous quantifications of the SEP violation. Predictions of “boiling oceans”, which contradict existing data, do not arise in the present model. (For the predictions of the Brans–Dicke theory see ref. 16.)

3-K Black-body radiation

The black-body radiation has long been considered of crucial importance to SEP violating theories. One important consideration must be stressed—*a priori*, it is not known whether an SEP violation requires the standard particle conservation law be changed and if so, whether photons and massive particles are equally affected.

The first theory implementing an SEP violation, that of Jordan¹⁷, made no predictions on this problem, thus leaving the question to be settled by observational data. In 1968, Honl and Dehnen¹⁸ showed that a non-constant photon number N_γ would be incompatible with the 3 K black-body spectrum. Whereupon Jordan¹⁹, acknowledging the impossibility of reconciling his theory with variable N_γ , suggested that the other version of his theory with constant N_γ be adopted. (This coincides with the Brans–Dicke theory.)

Perhaps unaware of the 1968 results, Dirac in 1972, 1973 and 1975 stated^{20–22} that a variable N_γ cannot be reconciled with the 3 K radiation. The same conclusion was reached by Canuto and Lodenquai²³ in 1977 in an attempt to test an SEP violation by the use of observational data. In 1978, again unaware of the 1968 work, Steigman²⁴ arrived at the same conclusion as Honl and Dehnen, namely that a non-constant N_γ is not compatible with the 3 K radiation.

Thereafter Canuto and Hsieh proposed two alternative solutions of the 3 K problem. In 1978, it was shown²⁵ that if instead of the gauges $G \sim \beta_a^{-1}$, ($G \sim t^{-1}$) previously used until then by Dirac, Jordan and so on, one chose (1) $G \sim \beta_a^{-2}$ together with (2) $\epsilon_\gamma \sim \nu$ and (3) $T \sim R^{-1}$, the resulting N_γ was constant and that the 3 K radiation does not exclude a violation of the SEP. However, since the necessary conditions (1)–(3) were assumed, not derived from a complete photon theory, it could not be claimed that the 3 K problem had been solved. In 1979, a complete photon theory was presented⁴ in which the propagation equation, the single particle relations and the thermodynamic relations were all derived in a consistent manner. The result was that the 3 K radiation is compatible with an SEP violation provided (1) $N_\gamma \sim \beta_a^{-1}$, (2) $\epsilon_\gamma \sim \nu/\beta_a$ and (3) $T \sim \beta_a^{-1} R^{-1}$.

In 1982, however, it was first realized¹ that a violation of the SEP demands $g = 2$. Since the photon theory of ref. 4 has been shown here to be valid for $g = 1$, (in spite of the original belief that it held for any g), the theory is no longer tenable. Unlike Jordan's theory, the 1979 photon theory has to be abandoned, not because of observational reasons but because of theoretical consistency requirements.

In the theory presented here, we have shown that N_γ remains constant for any g and that for the required value $g = 2$, all photon relations are identical with the standard ones. Photons are therefore unaffected by an SEP violation, and the 3 K radiation imposes no constraints on our formulation of the SEP violation.

Finally, we note that both Jordan's theory with variable N_γ (both N_γ and N_p), and that of Brans–Dicke with both N_p and N_γ constant, are not viable, although for different reasons. Our theory, with N_γ = constant, but $N_p \neq$ constant, has thus far not encountered such difficulties.

Nucleosynthesis

The nucleosynthesis of light elements has been a most valuable tool to probe the early Universe²⁶, and attempts have been made to use the ^4He abundance to set limits on the violation of the SEP, expressed phenomenologically as a variable G .

The most recent study²⁷ indicates that if the shape of β_a as a function of t determined from the matter era is extrapolated to the radiation era, unacceptable values of ^4He are obtained. Indeed, the nucleosynthesis data demand an almost constant β_a during the radiation dominated era, and for good reasons. The function β_a represents a cosmological influence on local physics^{1,2}. The dynamics of β_a is therefore determined by the global structure of the Universe which in turn is governed by the energy density (and pressure) of matter and radiation. Since matter is affected by β_a , equations (12)–(14), while radiation is not, it is plausible that during the matter-dominated era, the rate of variation in β_a is comparable with the rate of expansion—that is $\dot{\beta}_a/\beta_a \sim 1/t$, or $\beta_a \sim t^{-n}$, with $n \sim 1$. However, during the radiation era, such a variation is expected to have been considerably smaller, that is, $\dot{\beta}_a/\beta_a \ll 1/t$, since the dynamical part played by matter was negligible. In such a case, the value of β_a during the short radiation period would not have differed significantly from, say, the last ‘matter-dominated’ value at decoupling. Indeed, the main result of ref. 27 is that during the radiation era $\dot{\beta}_a/\beta_a \ll 1/t$, that is $\beta_a \sim t^{-n}$, $n \ll 1$.

We shall therefore propose that during the radiation era, β_a was essentially constant and investigate the consequences for nucleosynthesis. Using the standard framework, Olive *et al.*²⁸ have recently analysed the dependence of the calculated abundances on the most relevant parameters namely, (1) the baryon to photon number ratio $\eta = n_B/n_\gamma$ which in standard cosmology is equal to its present value $\eta_0 = \rho_{B0} 10^{22} (2.7/T_0)^3 / 6.64$, where ρ_{B0} is expressed in g cm^{-3} , (2) the neutron half life $\tau_{1/2}$, and (3) a speed-up factor ξ , defined by $\dot{R}/R = \xi(\dot{R}/R)_{\text{standard}}$.

In the SEP violating framework, a constant β_a affects nucleosynthesis in two ways. Because of $N_p \sim \beta_a$, we have $n_B \sim \beta_a R^{-3}$, while $n_\gamma \sim R^{-3}$. Therefore $\eta = \eta_0 \beta_a$. Furthermore, the RW equation⁵, with $G\beta_a^2 = \text{constant}$ and $k = 0$ becomes

$$\frac{\dot{R}}{R} = \beta_a^{-1} \left(\frac{8\pi G_0}{3} \rho_\gamma \right)^{1/2} = \beta_a^{-1} \left(\frac{\dot{R}}{R} \right)_{\text{standard}}$$

so that β_a^{-1} plays the part of a speed up factor ξ .

In Fig. 1 the calculated ^4He (Y) and D (D) abundances are plotted as a function of η_0 , for the standard ($\beta_a = 1$) and the present theory. In both cases, $N_\gamma = 3$ and $\tau_{1/2} = 10.6$ min. The standard model ^4He and D abundances were taken from refs 28, 29 respectively. The ^4He and D abundances corresponding to the present model were calculated from Fig. 2a of ref. 28 using $\xi = \beta_a^{-1}$, and $\eta = \eta_0 \beta_a$ and from Table 1 of ref. 29 (the D abundance is a function of $\eta/\xi = \eta_0 \beta_a^2$, ref. 29). We first consider the results of the standard case. Determinations of the ^4He abundance Y in recent years have limited the uncertainty to the range $0.25 \geq Y \geq 0.20$, corresponding to $4.5 \times 10^{-11} \leq$

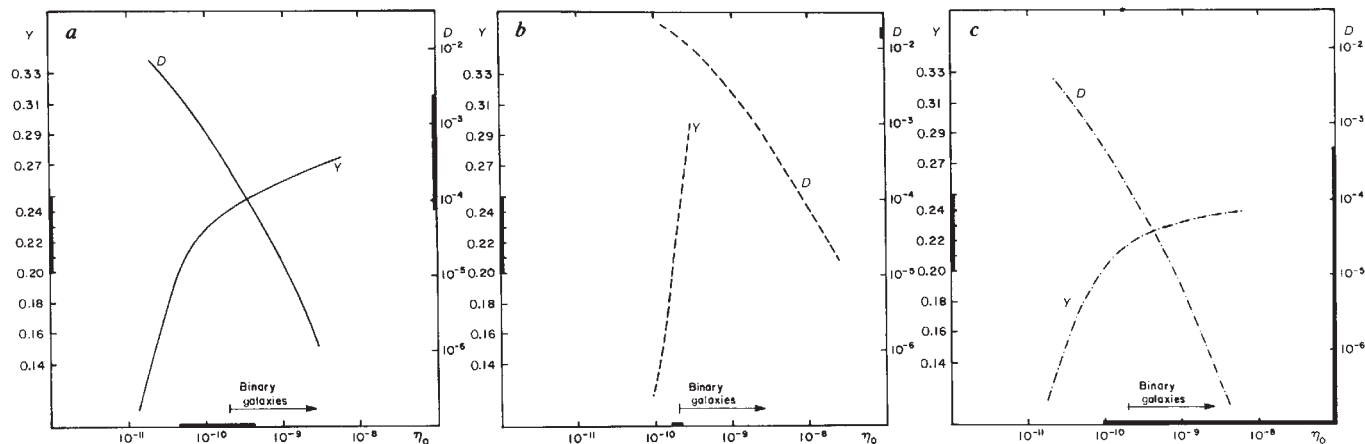


Fig. 1 Predicted helium (Y) and deuterium (D) abundances by mass. Given the range of observed values of Y , one determines the corresponding baryon to photon number ratio η_0 . This in turn is projected under the D curve and a D abundance is arrived at, which is then compared with observations. If one accepts the most recent D abundances $7.0\text{--}3.4 \times 10^{-5}$ (ref. 42) it is seen that the $\beta_a = 1.2$ case fits the data better than the standard case $\beta_a = 1$. *a*, $\beta_a = 1$; *b*, $\beta_a = 0.2$; *c*, $\beta_a = 1.2$.

$\eta_0 \leq 4 \times 10^{-10}$. This in turn implies that the D abundance must be $8 \times 10^{-5} \leq D \leq 2.5 \times 10^{-3}$. The presently observed D abundance is estimated³⁰ to be in the range $1\text{--}4 \times 10^{-5}$, an interval which may still be consistent with that given above since it must be a lower limit because D is in fact only destroyed by stellar processes. The quantification of the destruction is difficult. On the one hand, Rana³¹ has concluded that the primordial D abundance must have been $\sim 6 \times 10^{-5}$, implying that the standard framework is inconsistent. On the other hand, Yang *et al.*³² and Steigman³⁰ have argued that since D is converted into ^3He , the combined $D + ^3\text{He}$ should not be less than its present value. This leads to $\eta_0 \geq 2 \times 10^{-10}$, which in turn corresponds to a D abundance $\leq 2.5 \times 10^{-4}$, in agreement with the range previously determined. Even if this second solution is accepted, there may be a further problem. Recent data^{28,33} on the ^4He abundance have suggested values as low as 0.22, implying a D abundance $> 1.4 \times 10^{-3}$, a value which may be difficult to accommodate^{34,35}. (Note that the lower the ^4He abundance, the higher the D abundance.)

Finally, another potential source of inconsistency stems from the constraint²⁸ $\eta_0 \geq 2 \times 10^{-10}$, reached by considerations of dynamics of binary galaxies and small groups of galaxies. This value implies $Y \geq 0.24$, in contradiction with $Y = 0.22$, should this last value be confirmed by future observations. (Here too, a solution has been proposed^{28,30}: massive neutrinos, which by contributing to the masses of galaxies would allow a lower value of η_0 due to baryons and so a lower Y).

In conclusion, while it is premature to claim that inconsistencies have been found, it may turn out to be difficult to accommodate Y , D and the constraints of galaxy dynamics within the standard SEP conserving framework.

In the present framework, and on the basis of available lunar data, suggesting $\beta_a > 0$, we assume that β_a is monotonically increasing during the matter-dominated era. We have suggested a constant β_a in the radiation era. If β_a behaves monotonically during the radiation to matter transition period, then the constant value must be less than its present value and presumably not very different from its value at the transition time. It is, however, possible that the change of scenario during the transition period and the different behaviour of matter and radiation as a function of β_a , may have caused a non-monotonic behaviour during the transition period, thus allowing for a constant greater than unity value throughout the radiation dominated epoch. The implications for nucleosynthesis of these two alternatives were examined by considering two representative values of β_a , namely $\beta_a = 0.2$ and $\beta_a = 1.2$ (see Fig. 1).

$\beta_a = 0.2$. Repeating the same arguments as in the standard case, we see that the inconsistency between $\eta_0 \geq 2 \times 10^{-10}$ (binary galaxies) and $Y = 0.23$, no longer exists. The predicted value of D would be about 10^{-2} , a factor of 10 larger than the

value predicted by the standard framework if indeed $Y = 0.23$. **$\beta_a = 1.2$.** In this case, all the demands for low Y , D and η_0 (binary galaxies) can be satisfied and no inconsistencies arise. For $Y = 0.22\text{--}0.23$, η_0 is required to lie between $2\text{--}6.5 \times 10^{-10}$, in agreement with the limit contained from binary galaxies. At the same time, D would be $0.1\text{--}1.5 \times 10^{-5}$, again in agreement with the present data.

Comparison with previous schemes

Two features that differentiate our framework from previous SEP violating schemes are important.

First, they generally assume that (in AU) a violation of the SEP will affect only gravitational physics while leaving all non-gravitational relations unaffected. In the present theory, gravitational physics is affected by β_a but so are the non-gravitational many-body relations for massive particles; for massless particles, all relations are independent of β_a . (This results in a different G dependence of several of the tests, leading in general to a weaker dependence on G , see equation (17)).

The second, and perhaps more important difference is that the present theory allows $\beta_a/\beta_a \sim \Lambda H_0$, with $\Lambda \sim 1$, which is a violation of the SEP of the order of the Hubble constant, as expected on physical grounds for a violation of cosmological origin. By contrast, $\Lambda \sim 1$ cannot be achieved in other SEP violating theories, where it turns out that $|\Lambda| \leq 10^{-3}$. (In the Brans-Dicke theory²⁶, $\Lambda \sim (\omega + 2)^{-1}$ and³⁶ $\omega > 500$; in the Rosen theory³⁷, $\Lambda \sim \alpha_2$ and³⁸ $|\alpha_2| \leq 10^{-3}$).

Conclusions

We have attempted to test a possible SEP violation against already existing data, in the hope of providing an incentive for a direct experimental test. To that end, we have constructed a theoretical framework in which the SEP violation is represented by a function β_a treated phenomenologically, that is not provided by the theory itself, much as viscosity or heat conduction coefficients are treated in classical fluid dynamics. The implications of β_a on gravitational and non-gravitational phenomena are then checked against observational data ranging from astrophysics to geophysics and limitations on the variability of β_a arrived at.

Specifically, we have constructed a scale covariant (unit independent) formalism⁷, a mathematical procedure that *per se* does not introduce new physics, in much the same way that coordinate covariance requirement does not^{39,40}. In our case the physical input occurs when we define the properties that characterize gravitational as different from atomic units. (See ref. 1 for the definitions of the two units.)

In our previous paper¹, we showed that an SEP violation occurs when atomic and gravitational clocks run at different rates, which occurs provided that (1) the relation between the

function β_a and the gravitational coupling G is $G\beta_a^2 = \text{constant}$ and that (2) the equations of motion of microscopic particles are different from those of macroscopic bodies. This last property implies that one- and many-body systems respond differently to an SEP violation. In fact, while one-particle relations are unchanged, many-body relations for massive particles depend on β_a (equations (12) and (14)).

Our present treatment of massless particles under an SEP violation shows that the photon number N_γ is conserved and that because of the relation $G\beta_a^2 = \text{constant}$, all photon relations (single and many-body) are unaffected by β_a —they coincide with the standard expressions. The physical implication of this result is that a system of free photons cannot reveal the possible existence of an SEP violation, a result important for the study

of the 3 K black-body radiation and nucleosynthesis in the radiation dominated era.

Using the earlier results for massive particles¹, together with those obtained here, our compatibility tests ranging from nucleosynthesis to the radius of the Earth 400 Myr ago⁸, show that all the data we have analysed are consistent with an SEP violation of the order of the Hubble constant during the matter dominated era, $\beta_a/\beta_a \sim 1/t$, and with $\beta_a/\beta_a \ll 1/t$, during the radiation dominated era.

Thus our conclusion that an SEP violation while not demanded is compatible, will hopefully stimulate a direct experimental search using the best data available—the Viking data^{2,3,41}.

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Embryonic lethal mutation in mice induced by retrovirus insertion into the $\alpha 1(I)$ collagen gene

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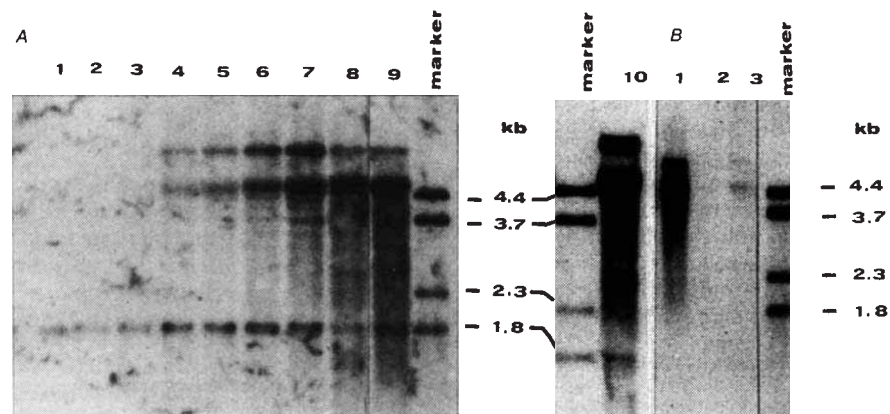
Experimental insertion of a retrovirus into the germ line of mice has resulted in an embryonic recessive lethal mutation. Integration of the proviral genome occurred at the 5' end of the $\alpha 1(I)$ collagen gene, leading to a complete transcriptional block. Developmental arrest of embryos homozygous at the mutated allele coincides with high expression of the gene in normal embryogenesis. Insertion mutagenesis by retroviruses may offer a general approach to the identification and isolation of genes which are transcriptionally active during mammalian development.

THE phenotypic analysis of experimentally induced or spontaneous mutations has long been the subject of developmental genetics in the mouse¹. Lethal mutations have had a significant role in identifying pleiotropic effects of presumably single genes on complex developmental processes. Examples of such genetic systems are the well-studied *T* locus², the albino deletions³ or mutations affecting fetal haematopoiesis⁴. This descriptive approach, however, has its limitations in elucidating the underlying developmental defect on a molecular basis, as a randomly mutated gene or its gene product is difficult to identify. An alternative approach to induce mutations by transposable elements, which permits the isolation of the mutated gene by using the element as a tag for molecular cloning, has been used successfully in prokaryotes⁵, yeast⁶ and *Drosophila*⁷.

We have used retroviruses as insertion mutagens to study gene regulation in early mouse development. The Moloney

leukaemia virus (M-MuLV) genome was inserted into the germ line by exposing early mouse embryos to virus⁸⁻¹⁰ or microinjecting cloned DNA into zygotes¹¹. Most of the resulting sub-strains of mice (Mov-1 to Mov-13 strains) each carry a single provirus at a different mendelian locus¹⁰ and the Mov-14 strain carries a tandem array of multiple proviral genomes¹². Virus integration at 13 different loci did not lead to a recognizable mutation in the respective animals. Virus insertion at the *Mov-13* locus, however, resulted in a recessive lethal mutation and early embryonic death¹³. All embryos homozygous for viral integration at this locus (*Mov-13/Mov-13*) were arrested in development between days 11 and 12 and died between days 13 and 14 of gestation. Using the virus as a probe, the genomic region of virus insertion was molecularly cloned and shown to represent a unique DNA sequence (ref. 13 and K.H., unpublished). We describe here the activation of the *Mov-13* locus

Fig. 1 *A*, *Mov-13* locus expression during mouse embryogenesis. C57BL/6 embryos from days 10 to 18 of gestation were isolated (the day of detection of the copulation plug is day 1). Several embryos from each day were pooled and total cellular RNA was prepared according to the method of Auffray⁴³. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose (Collaborative Research) chromatography as described by Aviv and Leder⁴⁴. After glyoxylation the RNA was electrophoresed in a 1% agarose gel, transferred to nitrocellulose paper⁴⁵ and hybridized first to the 14-kb *Eco*RI fragment corresponding to the *Mov-13* locus (Fig. 6; ref. 13) and rehybridized with a rat α 1-tubulin probe¹⁴ which gives rise to a 1.8-kb RNA band. Lanes 1–9, total RNA from day-10 to day-18 embryos, respectively; lane 10, total RNA from a newborn animal. *B*, Single embryos derived from heterozygous parents (δ *Mov-13*/wt $\times$ δ *Mov-13*/wt) were removed. DNA was extracted from each individual placenta to genotype the embryos as described in Fig. 3 legend and ref. 13. Poly(A)⁺-selected RNA was extracted from each embryo and analysed by Northern blotting as described above. Lane 1, RNA from day-15 wild-type embryo; lane 2, RNA from a homozygous day-12 embryo; lane 3, RNA from a day-12 wild-type embryo. The marker is a restriction enzyme digest of pBR322.



during embryogenesis, its tissue-specific expression in cells of different species and the derivation of tissue culture cells which are homozygous for the mutated gene. Our observations led us to screen the available clones of extracellular matrix genes and to identify the *Mov-13* locus as one of the most actively transcribed genes of the vertebrate body.

Mov-13 locus expression

To investigate whether M-MuLV in *Mov-13* mice had become integrated into an actively transcribed cellular gene, RNA was extracted from normal mouse embryos of different gestational ages or from organs of adult mice. A 14-kilobase (kb) *Eco*RI fragment representing the virus pre-insertion site, designated as the *Mov-13* locus, was cloned (ref. 13 and K.H. *et al.*, in preparation) and used as a probe for Northern blot analysis. Figure 1*A* shows that no *Mov-13* locus-specific RNA was detected in embryos at days 10 and 11 of gestation (lanes 1, 2). At day 12, two weak *Mov-13* locus-specific RNA bands of 5.2 and 6.5 kb appeared (lane 3) which were better seen when poly(A)⁺ instead of total RNA was analysed (compare Fig. 1*B*, lane 3). Increasing concentrations of these two RNA species were seen during subsequent gestational stages (lanes 4–9) and high concentrations were also found in newborn animals (lane 10). The filter was rehybridized with an α 1-tubulin probe, resulting in a band of 1.8 kb expected for tubulin mRNA¹⁴ in embryo RNA from all gestational ages. Different organs of adult animals showed low or no detectable levels of *Mov-13* locus-specific RNA (data not shown).

Embryos homozygous for viral integration at the *Mov-13* locus were arrested in development at day 12 and were dead at days 13 or 14 of gestation. To study whether M-MuLV integration disrupts *Mov-13* locus expression *in vivo*, heterozygous animals were mated, DNA was extracted from each individual placenta and the genotype of the respective embryo was determined as described in Fig. 3 legend and ref. 13. Figure 1*B* shows a Northern blot of poly(A)⁺-selected RNA from an embryo homozygous for virus integration at the *Mov-13* locus or of wild-type (wt) genotype (lanes 2, 3). While the *Mov-13* locus-specific RNA bands can be detected in the normal wild-type embryo, no RNA was expressed in the homozygous embryo.

The results in Fig. 1 show that *Mov-13* locus is developmentally regulated. Expression began at day 12 of gestation and increased sharply during subsequent fetal development. In adult animals, no or low expression of the gene was detected. In contrast, embryos homozygous for virus integration were unable to express the gene and were arrested in development between days 11 and 12 of gestation, at the time when the *Mov-13* locus becomes activated.

Mov-13 locus characterization

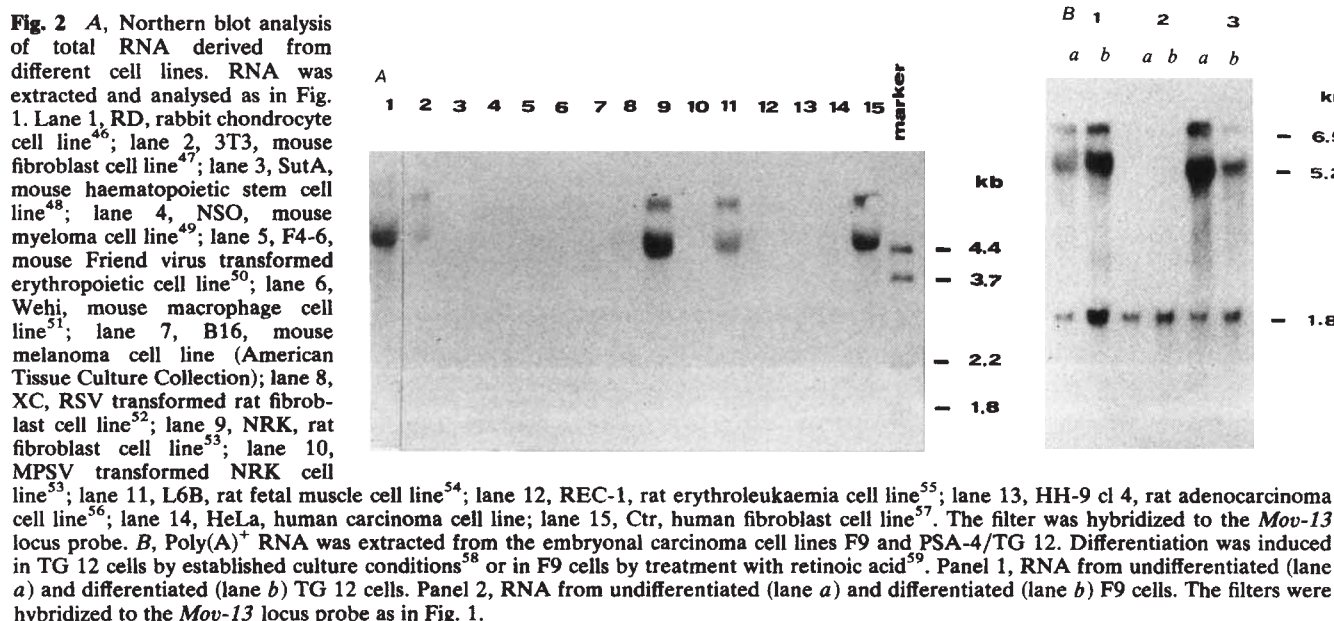
To characterize the tissue specificity of *Mov-13* locus expression, RNA was extracted from several established cell lines and analysed. Figure 2*A* summarizes the results obtained with different cell lines of mouse, rat, rabbit and human origin. High *Mov-13* locus expression was found in the fibroblast-like cell lines 3T3 and NRK, the human cell line Ctr (lanes 2, 9, 15), and in the rabbit chondrocyte cell line RD and the rat myogenic cell line L6B (lanes 1, 11). In contrast, the haematopoietic cell lines SutA, NSO, F4–6, Wehi-3 and REC-1 (lanes 3–6, 12), the mouse melanoma cell line B16 (lane 7), two epithelial cell lines HH-9 cl 4, a rat adenocarcinoma line and the human HeLa cell line (lanes 13, 14) did not express *Mov-13* locus-specific RNA. A strong reduction of *Mov-13* locus expression was found in cells transformed by sarcoma viruses. This was seen in XC cells, a Rous Sarcoma Virus (RSV) transformed rat fibroblast cell line (lane 8) and in NRK cells transformed with myeloproliferative sarcoma virus (MPSV) (compare lanes 9, 10).

To investigate *Mov-13* expression in embryonal cells, RNA was isolated from the embryonal carcinoma cell lines F9 and PSA-4/TG 12. Whereas F9 cells are known to have a restricted potential for differentiation into embryonal endoderm cells on cultivation in retinoic acid¹⁵, TG 12 cells effectively colonize many different tissues in chimaeric animals¹⁶. No *Mov-13* locus-specific RNA was found in undifferentiated F9 or TG 12 cells. When differentiation was induced, however, TG 12 cells expressed mRNA specific for the *Mov-13* locus, in contrast to F9 cells (Fig. 2*B*).

The results in Fig. 2 indicate that the *Mov-13* probe recognized a gene which is highly conserved during evolution, yielding strong hybridization signals in species as far apart as mouse and human. High expression of the gene appears to be restricted to mesoderm-derived fibroblast-like or myogenic cell lines and seems to be sensitive to transformation by sarcoma viruses. Other mesoderm-derived cell lines, like the haematopoietic cell lines, and cell lines originating from endoderm or ectoderm, or undifferentiated embryonal carcinoma cells, were negative for *Mov-13* locus expression.

Virus integration

To study whether cell lines homozygous for virus integration at the *Mov-13* locus were viable in tissue culture, individual embryos from parents heterozygous at the *Mov-13* locus (δ *Mov-13*/wt $\times$ δ *Mov-13*/wt) were trypsinized at day 12 of gestation, that is, before embryonal death, and plated on tissue culture dishes. To derive permanent cell lines, part of the cultures were transformed with Simian virus (SV40). DNA was



isolated from secondary cultures or from transformed cell lines, cleaved with *Eco*RI and subjected to Southern blot analysis using the *Mov-13* locus 14-kb *Eco*RI fragment as a probe (compare Fig. 6 and ref. 13). This experiment permitted the genotyping of embryos, as DNA from normal parental mice (C57BL/6) yielded a single *Eco*RI fragment of 14 kb, while that from embryos heterozygous at the *Mov-13* locus yielded two fragments of 14 kb and 23 kb, and that from homozygous embryos a single fragment of 23 kb (compare ref. 13). The analysis of three individual embryo cultures in Fig. 3A showed that embryo 2 was homozygous for virus integration at the *Mov-13* locus, whereas embryo 1 was heterozygous and embryo 3 was of parental (C57BL/6) genotype. Among 16 individually tested cultures, 6 were of homozygous, 8 of heterozygous and 2 of parental genotype. These results indicated that cells homozygous for virus integration at the *Mov-13* locus were viable in culture when isolated before embryonic arrest.

RNA was extracted from individual secondary cultures or SV40-transformed cell lines to study expression of the *Mov-13* locus. Northern blot analysis with total RNA revealed the two characteristic RNA bands of 5.2 and 6.5 kb in secondary cultures or SV40-transformed cell lines derived from normal or heterozygous embryos (Fig. 3B, embryos 1 and 3). This RNA was not detected in cells derived from homozygous embryos (embryo 2). When the same filter was rehybridized with an α 1-tubulin probe¹⁴, equal amounts of the expected tubulin mRNA band of 1.8 kb were detected in all cultures. These results indicated that integration of M-MuLV at the *Mov-13* locus has led to a transcriptional block of a highly active gene by insertion mutagenesis. The gene product was not expressed in homozygous day-12 embryos (Fig. 1B) and it appeared crucial for mid-gestation development of the mouse, although it was not essential for cell growth in tissue culture.

The α 1(I) collagen gene

The results described so far have indicated that the *Mov-13* locus was abundantly transcribed during the second half of embryogenesis. High expression was restricted to fibroblastic, myogenic and chondrocytic cells and was found in differentiated derivatives of the embryonal carcinoma cell line TG 12. Furthermore, expression of the gene appeared to be sensitive to transformation by sarcoma viruses and survival of fibroblastic cells in tissue culture did not depend on *Mov-13* locus expression (Fig. 3). These observations suggested to us that the gene product may be secreted and represent a protein of the extracellular matrix. Both type I collagen and fibronectin are

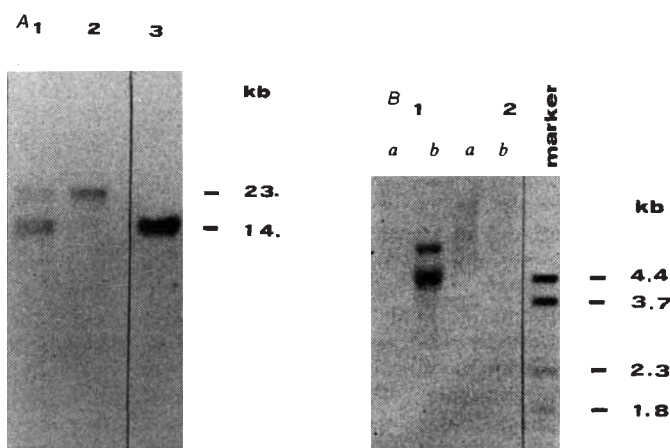


Fig. 3 A, Genotype of tissue culture cells derived from embryos of heterozygous parents (δ *Mov-13*/wt $\times$ δ *Mov-13*/wt). Single embryos were removed at day 12 of gestation; the cells were dispersed by treatment with trypsin and plated on tissue culture dishes. To derive permanent cell lines, part of the cultures were transformed with SV40. High molecular weight DNA was isolated as described elsewhere¹³. The DNA was digested with *Eco*RI (Boehringer-Mannheim) as recommended by the supplier, separated on 0.6% agarose gel and transferred to nitrocellulose filters⁶⁰. The cloned 14-kb *Eco*RI fragment representing the *Mov-13* preintegration locus (*Mov-13* locus probe) was nick-translated and hybridized to the filter as described elsewhere⁶¹. Lane 1, DNA from heterozygous (*Mov-13*/wt) embryo cells; lane 2, DNA from homozygous embryo cells (*Mov-13*/*Mov-13*); lane 3, DNA from normal embryo cells (wt/wt). The size of the *Eco*RI fragments before (14 kb) and after (23 kb) M-MuLV insertion is indicated. (For further details of the genotype analysis, see ref. 13.) B, Northern blot analysis of embryo cell lines with different *Mov-13* genotype. Total RNA was isolated from cells derived from an embryo heterozygous (panel 1) or homozygous (panel 2) at the *Mov-13* locus or from a wild-type (panel 3) embryo and analysed as in Fig. 1. RNA from secondary cultures (lanes a) or from SV40 transformed cells (lanes b) are shown. The filter was first hybridized to the *Mov-13* locus probe and subsequently to the α 1-tubulin probe as in Fig. 1.

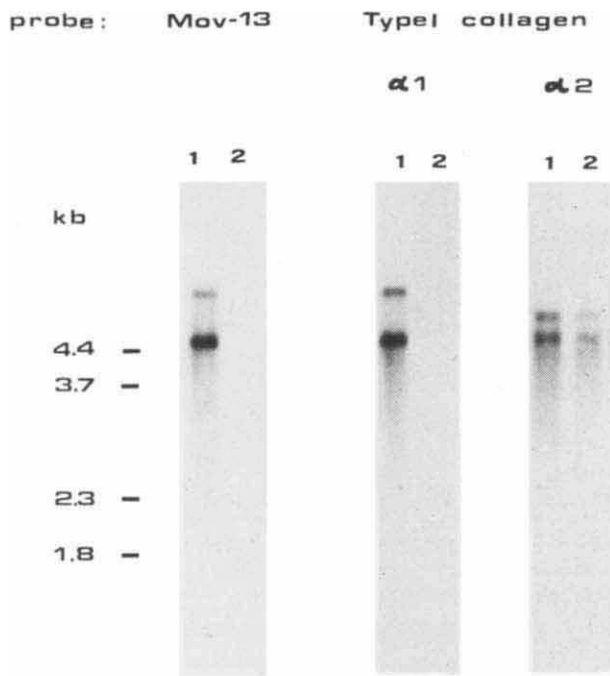


Fig. 4 Northern blot analysis with *Mov-13* locus and type I collagen probes. RNAs from day-12 embryo cell lines (Fig. 3) of wild-type (lanes 1) or *Mov-13* homozygous (lanes 2) genotype were isolated and analysed as described in Fig. 1. The filters were hybridized to the *Mov-13* locus probe (left panel), to the human $\alpha 1(I)$ cDNA probe Hf677²¹ (middle panel) and the human $\alpha 2(I)$ cDNA probe Hf32²² (right panel).

highly expressed in fibroblasts, are sensitive to transformation^{17,18} and are induced on differentiation of some embryonal carcinoma cells^{19,20}.

The $\alpha 1(I)$ and $\alpha 2(I)$ collagen genes are each transcribed into two mRNAs of 5–7 kb¹⁷, whereas a single mRNA of 8.8 kb is transcribed from the fibronectin gene¹⁸. We therefore tested whether the available collagen clones would hybridize with *Mov-13* locus mRNA.

RNA from cells derived from day-12 embryos that were homozygous for virus integration at the *Mov-13* locus or which were of parental C57BL genotype (compare Fig. 3) were hybridized to a *Mov-13* probe, to $\alpha 1(I)$ or $\alpha 2(I)$ collagen probes (Fig. 4). As described in Fig. 3B, the *Mov-13* probe gave two strong bands in RNA from normal but no hybridization with RNA from homozygous embryo cell lines (Fig. 4, left panel). When the RNA was hybridized to the $\alpha 1(I)$ probe, exactly the same hybridization bands were observed in normal but not in homozygous embryo cell lines (Fig. 4, middle panel). In contrast, two bands of different molecular weight were seen with the $\alpha 2(I)$ probe in embryos of both genotypes (Fig. 4, right panel). The collagen I cDNA clones of human^{21,22} and chicken^{23,24} origin gave the same hybridization patterns. These results strongly suggested that the *Mov-13* locus is identical with the collagen $\alpha 1(I)$ gene.

For further identification, DNA from heterozygous *Mov-13* and normal mice and human DNA were cleaved with *EcoRI* and subjected to Southern blot analysis. Figure 5 (left panel) shows that the *Mov-13* probe, as expected, recognized two fragments of 23 and 14 kb (lane 1) in heterozygous and only the 14-kb fragment in normal mouse DNA (lane 2). Human DNA yielded a single band of 22 kb (lane 3). The human $\alpha 1(I)$ cDNA clone hybridized to one band of 5.5 kb in mouse DNA (right panel, lanes 1, 2) and to the same fragment of 22 kb as seen with the *Mov-13* probe in human DNA (lane 3). The $\alpha 1(I)$ collagen probe only weakly recognized the 14-kb fragment of the cloned *Mov-13* locus (lane 4). In chicken DNA

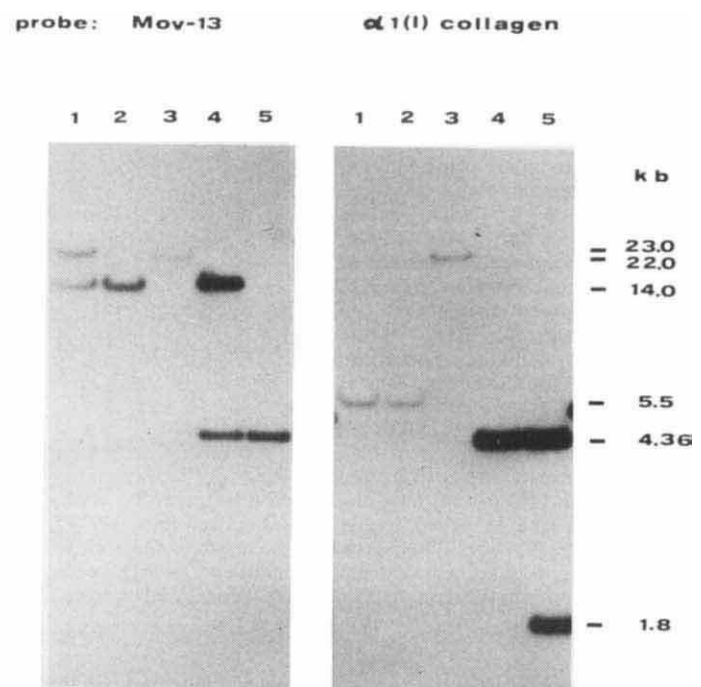


Fig. 5 Comparison of *EcoRI*-digested genomic and cloned DNAs hybridized either to a *Mov-13* locus or $\alpha 1(I)$ collagen-specific probe. 15 μ g of genomic DNA or 0.4 ng of plasmid DNA was digested with *EcoRI* and after electrophoresis transferred to nitrocellulose as in Fig. 3. Lanes 1, DNA from a *Mov-13*/wt mouse; lanes 2, DNA from a wild-type C57BL mouse; lanes 3, human DNA; lanes 4, DNA from the plasmid containing the 14-kb *EcoRI* fragment corresponding to the *Mov-13* locus; lanes 5, human $\alpha 1(I)$ collagen cDNA clone Hf677²¹ containing a 1.8-kb *EcoRI* fragment homologous to the 3' end of human $\alpha 1(I)$ mRNA. The left panel was hybridized to the *Mov-13* locus probe and the right panel to the human $\alpha 1(I)$ probe. Indicated are the sizes for the *EcoRI* fragments recognized by the *Mov-13* locus probe: 14 kb for the virus preinsertion site, 23 kb after integration of M-MuLV (compare ref. 13) and 22 kb in the human DNA. Hybridization with the cDNA clone gave rise to a fragment of 5.5 kb in mouse, 22 kb in human and 1.8 kb in $\alpha 1(I)$ plasmid DNA. The 4.36-kb fragment corresponds to pBR322.

both the *Mov-13* locus probe and the chicken $\alpha 1(I)$ collagen probe recognized the same *EcoRI* fragment of ~30 kb (ref. 22; data not shown). The interpretation of these results is outlined in Fig. 6.

Monson *et al.*^{25,26} have cloned a 5.5-kb fragment from mouse DNA using an $\alpha 1(I)$ chicken probe. We detected this fragment in mouse DNA (Fig. 5, lanes 1, 2) with the $\alpha 1(I)$ human probe but not with the *Mov-13* probe. As the $\alpha 1(I)$ collagen cDNA clones used for this experiment represent the 3' half of the $\alpha 1(I)$ mRNA in chickens²³ as well as humans²¹, the 14-kb *EcoRI* fragment we have cloned should represent genomic $\alpha 1(I)$ sequences 5' of the 5.5-kb fragment cloned by Monson *et al.*²⁵. The weak hybridization of the $\alpha 1(I)$ probe to the *Mov-13* plasmid (Fig. 5, lane 4) suggests that only a small part of the cDNA clone overlaps with the *Mov-13* clone. In contrast to mouse DNA, both the $\alpha 1(I)$ and *Mov-13* probes anneal to a single *EcoRI* fragment of 22 kb in human DNA (lane 3; compare ref. 22) or to a 30-kb fragment in chicken DNA.

Using different subfragments from the cloned *Mov-13* locus as hybridization probes for RNA blots, we have mapped the 5' end of the RNA to the same region at which the M-MuLV provirus is integrated and shown that the direction of transcription of the M-MuLV genome is opposite to transcription of the collagen gene (Fig. 6 and K.H., in preparation). This was confirmed by heteroduplex analysis (K.H. and H. Delius, in preparation).

Discussion

Integration of M-MuLV at the *Mov-13* locus resulted in insertion mutagenesis blocking transcription of one of the most abundantly transcribed genes of the vertebrate body. The developmentally regulated and cell-specific pattern of *Mov-13* locus expression and its sensitivity to transformation by sarcoma viruses led us to screen genes coding for proteins of the extracellular matrix and thus to identify the gene for the $\alpha 1$ chain of collagen type I as being interrupted by virus integration. The virus had inserted at the 5' end of the collagen gene, completely blocking its transcription. It has been shown that integration of retroviruses can lead to enhanced transcription of cellular genes in some instances²⁷. A direct downstream promotion of collagen sequences from the long terminal repeat viral promoter is unlikely, as the virus in *Mov-13* mice has inserted with its direction of transcription opposite to the transcription of the collagen gene (Fig. 6). The molecular basis underlying the block of transcription is unknown and is now under investigation.

So far, inactivation of cellular genes by retrovirus insertion has been documented in a few instances. In tissue culture, Varmus *et al.*²⁸ induced phenotypic reversion of transformed cells by infection with M-MuLV, and Kuff *et al.*²⁹ demonstrated the presence of A-type particle proviral genomes in mutated immunoglobulin genes. In animals two spontaneous mutations have been shown by Jenkins *et al.*^{30,31} to be associated with insertion of retroviral genomes into the germ line of mice. It is unclear, however, at what stage of development the virus has entered the germ line causing mutations in the respective mouse strains, nor have the mutated genes been identified.

In contrast to all other *Mov* substrains, the *Mov-13* strain was derived by microinjection of M-MuLV into day-8 post-implantation mouse embryos¹⁰. We have shown that virus replicates actively in all tissues of an infected embryo^{32,33}, and thus that it must have integrated into the primordial germ cell which gave rise to the first *Mov-13* animal, after day 8 of gestation. It is possible that this occurred at a stage when the $\alpha 1(I)$ collagen gene was already highly transcribed, which would suggest a preference of virus integration into transcriptionally active genomic regions. *In situ* hybridization experiments to test whether and at what stage primordial germ cells express the $\alpha 1(I)$ collagen gene are now being performed. If our assumption is correct that retroviruses preferentially integrate into actively transcribed DNA regions, exposure of early mouse embryos to virus might be a generally applicable method for mutating and identifying genes which are expressed during mammalian development.

The interaction of mesenchymal and epithelial cells has a decisive role in the induction and formation of organs during embryonal development^{34,35}. The extracellular matrix seems to be of fundamental importance for these tissue interactions; this consists of fibrous proteins, principally the collagens, embedded in a gel containing polysaccharides and glycoproteins^{35,36}. In mice, well-studied examples of interactions involving matrix proteins include the formation of the salivary gland³⁶ or the expression of different collagens in induction of metanephric mesenchyme^{34,37}. The *Mov-13* mouse strain may thus provide a model system for studying the function of a defined matrix protein in the early stages of development.

The first appearance of small amounts of type I collagen has been seen in day-9 embryos by immunofluorescence^{38,32}. Our data show that abundant transcription begins at day 12 of development and subsequently increases sharply. Homozygous *Mov-13* embryos develop normally up to days 11–12 and become arrested in development at the time when high collagen (type I) transcription starts. Histological examination of homozygous embryos has shown a general necrosis without malformations at day 12 of development (J. Löhler *et al.*, in preparation). The first cell necrosis becomes evident in the liver, which develops to the major haematopoietic organ at day 12 of gestation⁴. It therefore seems possible that the direct cause of the sudden embryonal death is not the block of collagen $\alpha 1(I)$ transcription *per se*. As the extracellular matrix is impor-

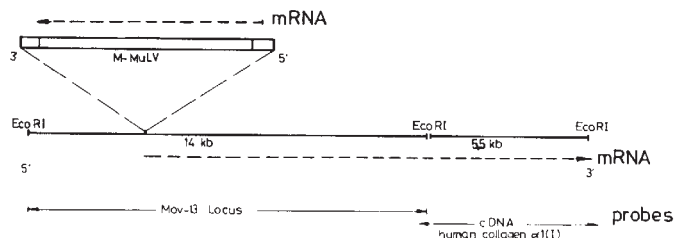


Fig. 6 Schematic map of the mouse $\alpha 1(I)$ collagen gene. Indicated are the two mouse *EcoRI* fragments of 14 kb and 5.5 kb, the site of M-MuLV integration and the direction of mRNA transcription for the M-MuLV and the collagen $\alpha 1(I)$ gene. The start site of the collagen transcript is mapped close to the integration site of the virus. The human cDNA clone corresponds mainly to the region where the 5.5-kb fragment is located.

tant in mesenchyme–epithelium interactions, inhibition of collagen type I may interfere with a complex chain of cell interactions which may involve cells such as those of the haematopoietic systems. An extensive analysis of *Mov-13* embryos should allow us to define the complex role of collagen during mammalian embryogenesis.

Type I collagen is a major structural protein of the body and is predominantly formed as a component of tendons, bone and skin of adult animals³⁹. Collagen fibrils are formed as trimers composed of two $\alpha 1$ chains and one $\alpha 2$ chain³⁹. In various genetic diseases⁴⁰ a structural defect in the gene⁴¹ or an altered relative concentration of the two collagen chains⁴² lead to severe symptoms in children or even death in the newborn. The availability of an animal model which allows us to study the collagen composition in animals with one or two $\alpha 1(I)$ genes being active may increase our understanding of the pathogenesis of human disease.

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Note added in proof: Recently we have obtained the human $\alpha 1(I)$ clone pg 1H-1 from Dr D. Rowe. This clone represents 5' as well as 3' sequences of the human gene. When annealed to DNA from heterozygous *Mov-13* or parental genotype mice, the expected three bands of 23 kb, 14 kb and 5.5 kb in *Mov-13*/wt DNA and two bands of 14 kb and 5.5 kb in wt/wt DNA were seen (compare Fig. 5, lanes 1 and 2). This confirms that M-MuLV has integrated into the $\alpha 1(I)$ collagen gene of *Mov-13* mice.

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mRNA sequences define an unusually restricted IgG response to 2-phenyloxazolone and its early diversification

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Light and heavy chain mRNAs of 15 anti-oxazolone antibodies were sequenced directly using synthetic primers. Hybridomas obtained 7 days after primary immunization show that they are of restricted heterogeneity. The majority expressed a single sequence with minor differences concentrated in the D region and joining boundaries D–J_H and V_κ–J_κ.

THE antigen binding specificity of an immunoglobulin molecule is determined by the amino acid sequences of the variable (V)-regions of the heavy (H) and light (L) chains. The diversity of V-region structures is vast, and complex even in an immune response directed to simple antigen (for example, hapten). This complexity has several levels. The first is generated by the somatic combinatorial integration of three sets of heavy chain (V_H , D and J_H) and two sets of light chain ($V_κ$ and $J_κ$) germ-line gene segments coding for the V regions^{1–4}. In the mouse there are four functional J genes for each chain, a larger number of D genes and a still larger number (100–300) of V genes. A second level of somatic diversification is thought to be due to point mutations^{5–7}, but may also include recombination and gene conversion⁸.

A better understanding of this problem requires a more detailed picture of the heterogeneity of the response to specific antigens. These studies have been directed towards the more homogeneous (idiotypic) responses against haptens or simple antigens. Hybrid myelomas made it possible to isolate and study the individual elements which contribute to the heterogeneity of these restricted responses. Amino acid^{9–11} and some nucleotide^{12,13} sequences of antibodies have demonstrated the importance of somatic diversification superimposed on the combinatorial integration of a restricted number of germ-line genes involved in these idiotypic responses. A better picture of the mechanisms requires an understanding of the early stages of the response and the pattern of subsequent diversification. This demands even simpler systems and more extensive studies. We have undertaken a sequence comparison of both light and heavy chains of 15 monoclonal antibodies from a primary response against the hapten 2-phenyloxazolone (phOx), obtained 7 days

after immunization. The response to this hapten is known to be of restricted heterogeneity in several mice and rat strains^{14,15}. Monoclonal antibodies obtained 7 days after primary immunization have a more narrow distribution of avidities than those obtained after 14 days¹⁶. The antibodies from the 7-day response have an affinity of the order of 10^{-7} mol l⁻¹, are mostly of the IgG1 class and 80% of them are recognized by a rabbit anti-idiotypic antiserum directed against the anti-phOx heavy chain. Their mRNA sequences disclose remarkable similarity, probably due to the expression of a single set of unmutated germ-line V_H and $V_κ$ genes, an unusual D gene, and $J_{κ5}$ and J_{H3} genes. Minor differences in the sequences are concentrated in the D regions and at the D – J_H and $V_κ$ – $J_κ$ joining boundaries. Five out of 23 BALB/c hybridomas tested were idiotype negative¹⁶. Two of them (NQ2-6.1 and NQ2-45.1) were taken at random for this study. The sequences of their heavy chains were from a different V_H family. The sequencing strategy is described in Fig. 1.

Restricted heterogeneity

The sequences listed in Figs 2 and 3 include hybridomas obtained using 7-day primary immunized animals in three independent fusions: NQ2 and NQ5 from BALB/c and NQ6 from DBA/2 mice. The majority of the anti-oxazolone antibodies have the same V_H and the same $V_κ$ or minor variations of these sequences (see Fig. 4 for a simplified version of the data). These therefore are likely to define germ-line sequences and we refer to them as V_H -Ox1 and $V_κ$ -Ox1. The only difference between V_H -Ox1 segments was a single (expressed) nucleotide in codon 64 of both DBA/2 hybridomas and this probably represents an allotypic marker of the V region. Although other examples defined by amino acid sequences have been described¹⁷, to our knowledge this is the first V -region

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Table 1 Amino acid sequence comparison of anti-arsonate and anti-oxazolone light chains

Residue no.	1-88	89	97	98-108
<i>Anti-arsonate:</i>				
HPR16.7	Prototype	Q Q G N S L P R T	Prototype	
HP93G7	—	—	M	—
HP1 123E6	— × —	— Y —	M	—
HP124E1	— × — × —	— K —	T	—
HP91A.3	— × — × — × —	— A —	—	—
<i>Anti-phOx:</i>				
NQ5-89.4	—	— T —	Y —	—

Anti-arsonate sequences taken from ref. 10. Lines indicate sequence identity. Crosses indicate location of amino acid sequence differences.

allelic marker defined at the mRNA level, and supports the suggested preference for non-silent nucleotide substitutions discussed below.

The remarkable stability of the V_H -Ox1 sequence does not support the idea that the switch of IgM to IgG induces somatic mutants⁷. A major role of the switch in the generation of mutants has been questioned before¹⁸. The accumulation of mutants is more likely to be time dependent, with a major role of antigen in their selective proliferation. Of the few IgM antibodies obtained at day 7, we selected the only one classified as possibly idiotype positive. Although its sequence is similar to V_H -Ox1, it differs in at least 21 codons, 11 of them expressed as amino acid substitutions. The allelic marker residue 64 is Ile rather than Met, and the sequence is almost identical to another germ-line gene which we will call V_H -Ox2 (see legend of Fig. 2).

The V_κ segments give a similar picture. Eleven out of 15 sequences seem to originate from the V_κ -Ox1 gene since the only detected difference was a single (expressed) nucleotide substitution (codon 55 of NQ5-61.1.2). In addition, two sequences (NQ2-45.10.4 and NQ2-6.1) are highly related to V_κ -Ox1. These two are more similar to each other than they are to the rest. They may be somatic mutants of V_κ -Ox1, or more likely derive from another germ-line gene. Both are associated with heavy chains not expressing the basic V_H -Ox1 sequence. Of specific interest is that over 80% of the codons where nucleotide substitutions occur, give rise to amino acid substitutions. A bias towards expressed nucleotide substitutions in V gene evolution has been observed by some workers^{19,20} but not by others (for example, ref. 21). Our results support the idea that evolutionary and probably somatic selection of V genes favours amino acid replacements over silent mutations.

It is important that NQ5-89.4 has a completely different V_κ and is idiotype positive. This was not surprising, since the idiotypic antiserum was prepared with isolated anti-Ox heavy chains reassociated to light chains from non-immunized animals¹⁶. Therefore the large proportion of a single V_κ sequence associated with the oxazolone V_H is not an artefact introduced by idiotypic selection. NQ5-89.4 has an affinity for oxazolone which is of the same order of magnitude as the average of 7-day anti-oxazolone monoclonal antibody (Fig. 4). Why then is this type of heavy-light chain combination so rare? We suggest that the unmutated genes give a low affinity antibody, whilst NQ5-89.4 contains critical mutations allowing this V_H - V_κ combination to exhibit a significantly improved affinity for the phOx (for example, glycine instead of arginine in the D segment).

The NQ5-89.4 V_κ is interesting in another way. Its deduced amino acid sequence is almost identical to anti-arsonate light chains²². It seems to differ from the anti-arsonate prototype by only two amino acid residues—at position 93 (a hot spot in anti-arsonate sequences) and at position 96, the V-J boundary, an arginine in all five anti-arsonates which is a tyrosine (the germ-line J_2 codon) in NQ5-89.4 (Table 1). Since the NQ5-89.4 avidity for oxazolone is within the 7-day average (see Fig. 4) this provides an interesting example of the combinatorial effect of heavy and light chains to produce different antibody specificities (see also ref. 23).

New D segment defined

All the D segments associated to V_H -Ox1 are remarkably similar and in some cases identical. It seems unlikely that such remarkable similarity could arise by random processes. A D gene or a family of related D genes are probably involved in coding for the D segments of the basic phOx antibody. Three families of D germ-line genes have been described and it has been suggested that they represent most, if not all D genes²⁴. None of the members of those gene families was homologous to the D regions described here. We conclude that the Ox idiotype D segments are probably coded by a hitherto undescribed germ-line gene(s).

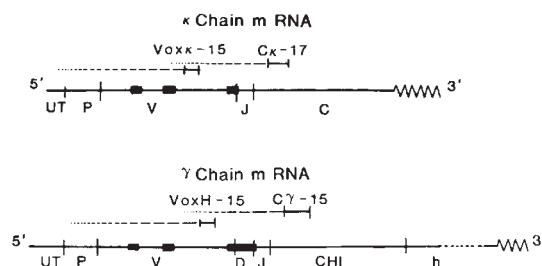


Fig. 1 mRNA sequencing strategy. Synthetic oligonucleotide primers designed to pair with defined bases within segments of mRNAs were used to initiate reverse transcription. Using dideoxynucleotides, specific stops in the cDNA can be generated and the nucleotide sequence determined by gel methods. The results are shown in Figs 2 and 3 and compared with a basic sequence of the oxazolone idiotype¹⁶. As the intention of this study was an overall view of the diversity of sequences, we made no extensive efforts to clarify some difficult residues. In most of these cases a likely assignment could be made. A question mark or a lower case letter was used to indicate this. In some cases the ambiguity was such that no assignment was made. These residues were left blank. Five synthetic oligonucleotide primers, complementary to specific sites on the mRNA were used in the direct sequencing technique. (1) γ -Chain primer (Cy-15), d(GGCCAGTGGATAGAC)¹⁶ hybridizes to the constant region 22–37 bases before the V region. Priming at this site enables all γ subclasses to be sequenced. (2) μ -Chain primer (C μ -15), d(GCTCTCGCAGGAGAC) hybridizes to the μ constant region between the first base of valine and the third base of serine codons (34–49 bases into the C region). (3) Heavy-chain oxazolone-idiotype primer (VoxH-15), d(GCTCTTGGAGTTGTC)¹⁶ hybridizes between the first base of aspartic acid 72 and the third base of serine 76 of the oxazolone idiotype V_H region. (4) Light-chain C primer (Ck-17), d(TGGATGGTGGGAAGATG)¹⁶ hybridizes between the third base of serine 116 and the first base of serine 122, 26 bases into the C₁ segment. (5) Light-chain oxazolone idiotype primer (Voxk-15), d(CCCACTGCCACTGAA)¹⁶ hybridizes between the first base of the phenylalanine 62 and the last base of glycine 66 of the oxazolone idiotype V_κ region. The sequencing procedure was essentially as described previously²⁹. cDNA synthesis directed by these primers was carried out using reverse transcriptase in the presence of 15 μ M ³²P-dXTP, 50 μ M dNTP, 20 μ M ddNTP, 0.4 μ M ddXTP (where XTP is either C or A and NTP are the other three) using 15–60 ng of primer and 4 units of enzyme per μ g pf poly(A)⁺-enriched RNA at 42 °C for 15 min. Total RNA could also be used at a 10-fold higher concentration.

[illegible]

Fig. 2 Immunoglobulin heavy-chain mRNA sequences (*V* regions) from anti-phOx hybridomas. The top line nucleotide sequence displays the Ox-idiotype basic sequence and *J*₃ germ-line gene sequences³¹. Dashes in the sequences indicate identity to the top line. A question mark indicates probable identity. Upper and lower case letters are used respectively to indicate established and probable differences. When no nucleotide assignment was made, the positions were left blank. When the nucleotide change causes a change in the deduced amino acid sequence, the amino acid change is marked above the codon. Two hybridoma lines marked (*) originate from DBA/2 mice, others are from BALB/c mice. The cell line marked (+) is an IgM secreting hybridoma, others IgG1. The hybridomas presented in this figure originate from three independent cell fusion experiments NQ2, NQ5 and NQ6, and further details of their derivation and properties are given in ref. 16. The sequence of the *V*_H segment of NQ2-45.10 is identical to the sequence of the germ-line gene *V*_{H101} except for the first nucleotide of Thr 84. The germ line is Ser (TCT) (ref. 36). We refer to this germ-line sequence as *V*_H-Ox2.

Most anti-PhOx hybridomas expressed the same V_H , D , J_H and V_K genes, presumably because amongst germ-line genes this is the best combination for the oxazalone binding. Preferential combinatorial expression could be the result of nonrandom events in the joining mechanism, or of selective proliferation of otherwise randomly generated combinations. For example, to form a good binding site, the V_K - $Ox1$ may require a leucine residue at position 96 which is only found in the germ-line J_5 gene. Hence the preference for J_5 . A light chain of the type NQ5-78.2.6 expressing V_K - $Ox1$ attached to J_4 occurs, but at a lower frequency; possibly because the germ-line phenylalanine of J_4 must be substituted by a leucine. Preferential com-

That imprecise integration could be a generator of diversity, was first recognized in light chains at the $V-J$ junction⁴. The

OX-IDIOTYPE KAPPA-CHAINS

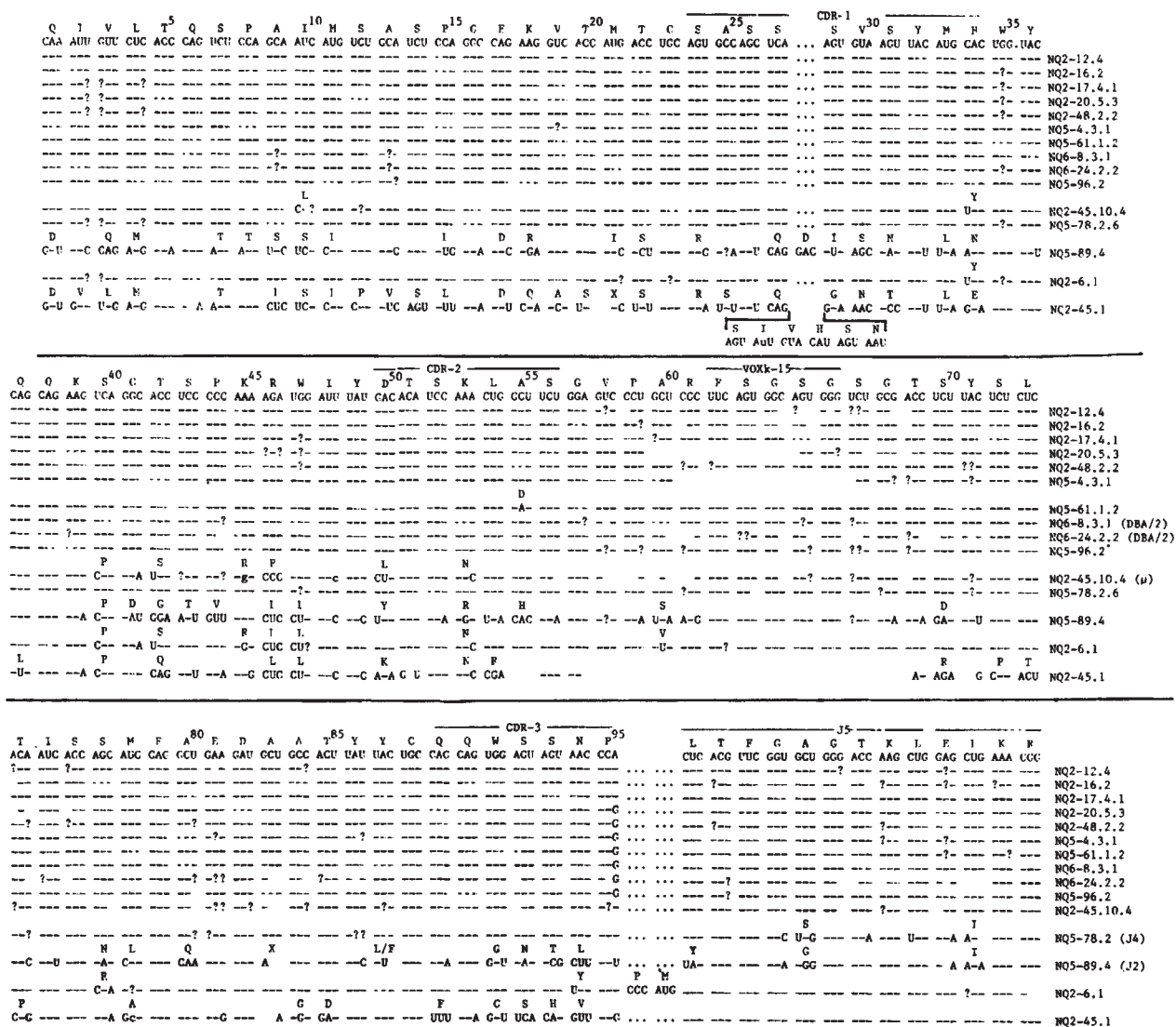
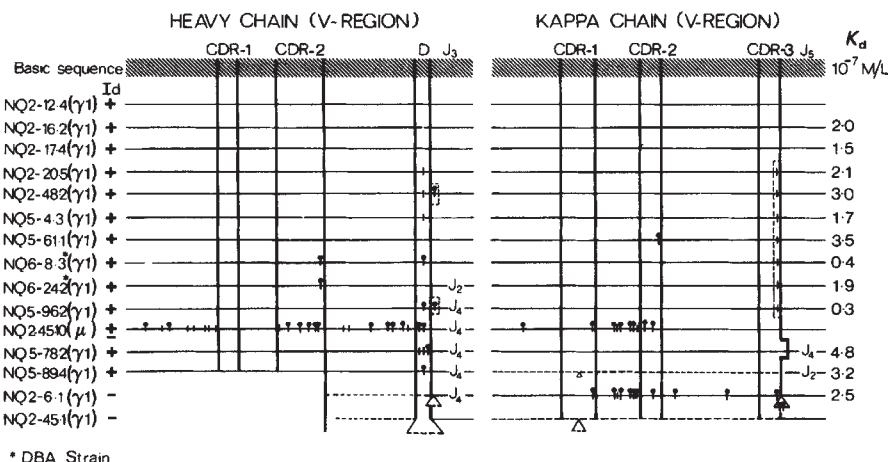


Fig. 3 Kappa-chain V-region mRNA sequences from anti-phOx secreting hybridomas. The cell lines are the same as in Fig. 2. The top nucleotide sequence is the basic Ox-idiotype kappa-chain mRNA sequence. For other details see Fig. 2 legend.

Fig. 4 Diagrammatic comparison of the mRNA sequences. The anti-oxazolone hybridomas were obtained using spleens from animals 7 days after primary immunization. The heavy chain class of the antibodies (in brackets) and the anti-idiotype reactivity (Id) are taken from ref. 16. Dissociation constants (K_d) were determined by the fluorescent quenching method as described³⁰ using purified monoclonal antibody (protein A-Sepharose columns) and 2-phenyl-5-oxazolone amino-caproyl¹⁴ as hapten. Full horizontal lines indicate sequence identity. Broken horizontal lines indicate extensive sequence differences. A vertical bar shows the position of codons containing nucleotide differences and a black circle indicates amino acid differences deduced from the mRNA sequences. The vertical divisions show the position of hypervariable regions (CDR). J_2 , J_4 indicate identity to the corresponding germ-line segment. Vertical broken lines indicate the suggested positions at which the nucleotides from the germ-line J segments or D segments may have been used during integration.



Quantized evaporation from liquid helium

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We report here the process for the evaporation of He atoms from the surface of liquid ^4He . It is a particularly simple quantum process: a phonon in the liquid with energy $\hbar\omega > E_B$, the binding energy, propagates to the free surface where it is either reflected or gives all its energy to an atom on the surface. This energy is used to overcome the binding forces and the balance, $\hbar\omega - E_B$, is carried away by the atom as kinetic energy.

The photoelectric effect, in which an electron is ejected from a metal by a photon, was discovered over 80 yr ago, yet the analogous process of the ejection of a single atom by a phonon has not been detected. We are familiar with the notion that atoms can leave liquids and solids to become free particles and we understand that these atoms must have an energy $\gg kT$ to overcome the binding energy of the condensed state, however, we have little idea as to how the atom acquires an energy which is far in excess of the average value. The unravelling of the microscopic processes for classical liquids and solids appears to be a formidable task but for some systems such as quantum liquids and solids, and the desorption of absorbed atoms from solids, the problem looks tractable.

In liquid ^4He the elementary excitations are phonons and rotons which are collective excitations of the atoms; the dispersion curve^{1,2} for these excitations are shown in Fig. 1. The single atom in the bulk liquid has no significance. The phonons have relatively long mean free paths which can, in certain circumstances, be many millimetres³. For example, if the liquid ^4He is cooled to a temperature ≤ 0.1 K the number of thermally excited phonons and rotons is so small that scattering from them is negligible. A phonon emitted into this cold liquid propagates until it either spontaneously decays or reaches an interface. The mean free path (λ) is limited by spontaneous decay and this is a strong function of the energy ($\hbar\omega$) of the phonon⁴. As $\lambda \sim \omega^{-5}$ the mean free path is short for phonons with energy E_B but at a critical frequency ω_c ($\hbar\omega_c \sim 9.5$ K) a remarkable change occurs in the energy dependence and the mean free path increases by $\sim 10^7$ and remains of the order of centimetres for higher energies. As the binding energy is $E_B/k_B = 7.15$ K (ref. 5) there is a range of phonons between 9.5 K and ~ 14 K which have enough energy to desorb a surface atom and have a long mean free path in the liquid. It is this combination which has allowed us to design an experiment which shows quantum evaporation.

It would not be surprising if we were able to show that a beam of phonons incident on the free surface of liquid ^4He liberates atoms but to show that it involves a single phonon excitation and a single atom in a one-to-one process requires gauging the energy of the incident phonon and measuring the kinetic energy of the liberated atom. To do this we use the dependence of the group velocities on the energy of the phonon and atom which are reasonably well known from the neutron-measured dispersion curve for liquid ^4He and the free atomic mass respectively. The experimental arrangement is shown in Fig. 2. A pulse of phonons is injected into the liquid ^4He by the heater and is collimated into a beam directed at the liquid-vacuum interface. Atoms liberated at the surface travel through the vacuum to a bolometer which detects them. The bolometer signal, $S(t)$ is captured on a transient recorder and then stored so that many repetitions can be averaged to achieve an acceptable signal-to-noise ratio. The distance (l) between the heater and bolometer is fixed but the separation (x) of the heater and liquid ^4He surface can be changed so that the overall propagation time (t) is also changed.

To establish the velocities of the phonons and atoms we use the minimum time (t_m) between the beginning of the heater pulse and the leading edge of the signal. If the group velocities of the phonons and atoms are $v_p(k_p)$ and v_a respectively, the time between the heater pulse and detected signal is

$$t = \frac{x}{v_p} + \frac{(l-x)}{v_a} \quad (1)$$

From conservation of energy we also have

$$\hbar\omega_p(k_p) = E_B + \frac{\hbar^2 k_a^2}{2m} \quad (2)$$

where the phonon energy $\hbar\omega_p$ is a function of the wavevector k_p , and k_a and m are the wavevector and mass of the He atom, respectively. The group velocities are given by

$$v_p = \frac{d\omega_p}{dk_p}, \quad v_a = \frac{\hbar k_a}{m} \quad (3)$$

values of $v_p(k_p)$ were found from $\omega_p(k_p)$ by fitting a polynomial to the neutron data in refs 1 and 2 and E_B is taken as the latent heat per atom. So for a phonon with a given k_p we find ω_p and v_p from the dispersion curve and calculate the kinetic energy of the atom from equation (3). Then from a measurement of x and l , the time for the corresponding signal can be calculated

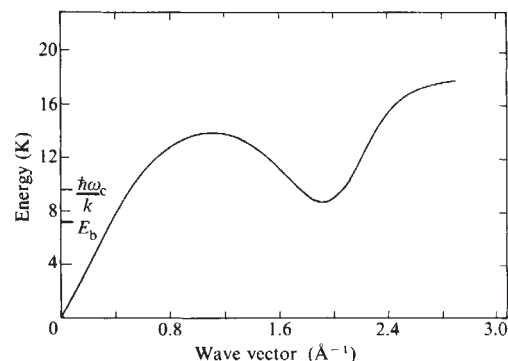


Fig. 1 The dispersion curve for excitation in liquid ^4He at the saturated vapour pressure at $T = 1.1$ K

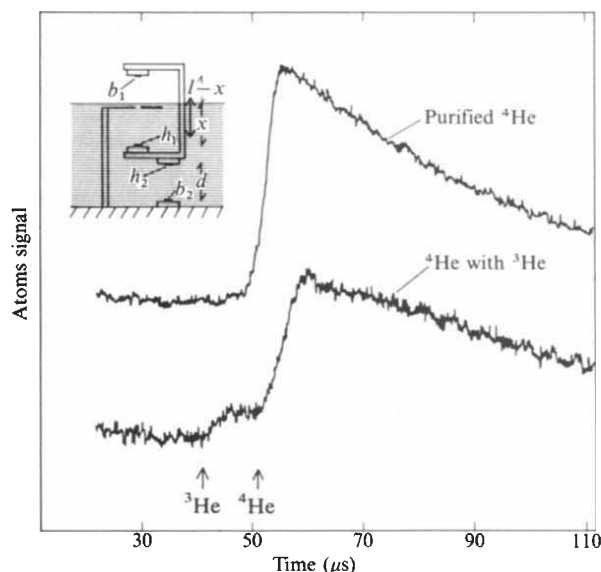


Fig. 2 The received signal as a function of time due to atoms liberated by phonons. The upper trace is the signal from only ^4He atoms and the lower one from both ^3He and ^4He atoms. The arrows indicate the calculated fastest times for this distance. Inset shows the experimental arrangement.

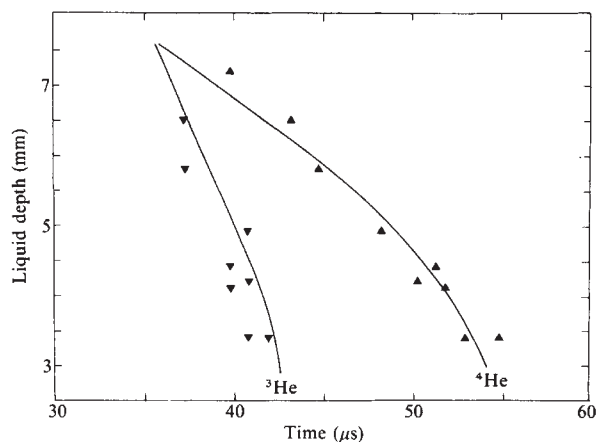


Fig. 3 The fastest time as a function of liquid depth. The total distance between heater and bolometer is 7.6 mm. The solid lines are calculated.

from equation (1); l is measured at room temperature and the small calculated correction for thermal contraction subtracted. The heater and bolometer are fixed to a carriage which can be moved vertically by a superconducting stepping motor so that different values of x can be selected. The collimator is fixed relative to the liquid surface. The height (d) of the carriage above the cell base is measured by the time of flight of low-frequency phonons which have a velocity of 238 m s^{-1} . The zero of this scale is found by moving the bolometer b_1 , just into the liquid. This point can be found with good resolution as the signal changes markedly when the bolometer is immersed in liquid ^4He .

The thin film heater h_1 produces a broad spectrum of phonon frequencies. Those phonons with $\omega_p < \omega_c$ rapidly decay to low frequencies and so reach the surface first, however, their energy is well below E_B . Those with $\omega_p > \omega_c$ reach the surface later and arrive in energy ordered sequence due to dispersion in the liquid ^4He . However, as ω_p increases, the energy given to the desorbed atom increases and hence the time of flight of the atom decreases. The combined result of these two effects means that for a given x , the total time, t for a signal due to monochromatic phonons to propagate between heater and bolometer is a function of the phonon frequency. Furthermore, $t(\omega_p)$ has a minimum value in the range $\omega_c < \omega_p < 14 \text{ K}$ and this time defines t_m . As the group velocities of the phonons and atoms are generally different then t_m is a function of x . Note that the frequency of the phonons which give rise to the signal at $t_m(x)$ also varies with x .

Values of t_m were found by calculating $t(\omega_p)$ for a given x and then finding the minimum in $t(\omega_p)$. Calculated values of t_m are shown in Fig. 3 for both ^3He and ^4He atoms. The values of E_B used in the calculation were 5.0 K (refs 5,7) and 7.15 K (ref. 5) for ^3He and ^4He respectively.

The measurements of t_m were made on purified ^4He (ref. 8) which had a ^3He content of 2 parts in 10^{10} . ^3He atoms go preferentially to the free surface of liquid ^4He when the number of ^3He atoms is low enough that less than a monolayer is formed⁸. The experimental cell had no sintered material above the liquid surface so from the surface area of the free liquid we estimate that the concentration of ^3He on the surface was $\sim 0.1\%$ of a monolayer. The liquid was cooled to $\leq 0.1 \text{ K}$ with a dilution refrigerator. At this temperature the vapour pressure above liquid ^4He is so low that the space is an excellent vacuum. Any liberated atom will move ballistically with a negligible chance of being scattered before it is detected. Typical signals are shown in Fig. 2 with arrows indicating the calculated minimum times for ^3He and ^4He .

After all the measurements were taken on the purified ^4He , a calculated amount of ^3He gas was admitted to the cell fill line and flushed down with ^4He gas. It took a few hours for the ^3He

to reach the cell and produce a measurable effect on the signal, so to ensure that equilibrium had been reached the experiment was left overnight. It was estimated that the ^3He coverage on the free ^4He surface was 23%. The received signal showed an earlier component which is as we expected because ^3He has both a smaller mass and lower binding energy than ^4He . The signal after the admission of ^3He is shown in Fig. 2 and may be compared directly with that for ^4He as all other variables have the same value.

The minimum times, t_m , measured from such data as in Fig. 2, are shown in Fig. 3. The good agreement between the calculated and measured values is immediately apparent and indicates that quantum evaporation of surface atoms by phonons is indeed taking place.

Thus, we have shown that phonons in liquid ^4He can evaporate both ^3He and ^4He atoms from the free surface of liquid ^4He . The interaction between the phonons and surface atoms is a one-to-one quantum process as has been conjectured by several authors⁹⁻¹¹ but has not hitherto been demonstrated. The energy of the phonon goes in overcoming the binding forces and the balance as kinetic energy of the free atom. Liquid ^4He , therefore, is the first liquid in which we can understand evaporation at the atomic level.

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Facular influences on the apparent solar shape

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Schatten and Sofia¹ have recently reconsidered the question of whether the 1966 solar ellipticity measurements² were seriously contaminated by excess brightness of faculae near the solar limb³⁻⁷. They considered several different functions for the variations of the facular contrast with position relative to the solar limb. With their own facular contrast function, Schatten and Sofia obtain only a small contribution of faculae to the 1966 apparent solar ellipticity, but with the Chapman function they obtain a substantial contribution. New observations of faculae during the summer of 1982 and a novel analytical technique determine a facular contrast which is constant or decreasing towards the limb, consistent with the Schatten and Sofia function but inconsistent with Chapman's function. We show here that the statistical analysis of the 1966 data⁸ supports this result. We disagree with the earlier conclusion¹, that with an acceptable facular contrast function one can obtain "an acceptable fit to the oblateness measurements" as a purely facular effect. For 20-30% of the observational days in 1966 only a few small, weak facular patches were present at the limb, but the ellipticity signal was present and it was not reduced in magnitude for those days.

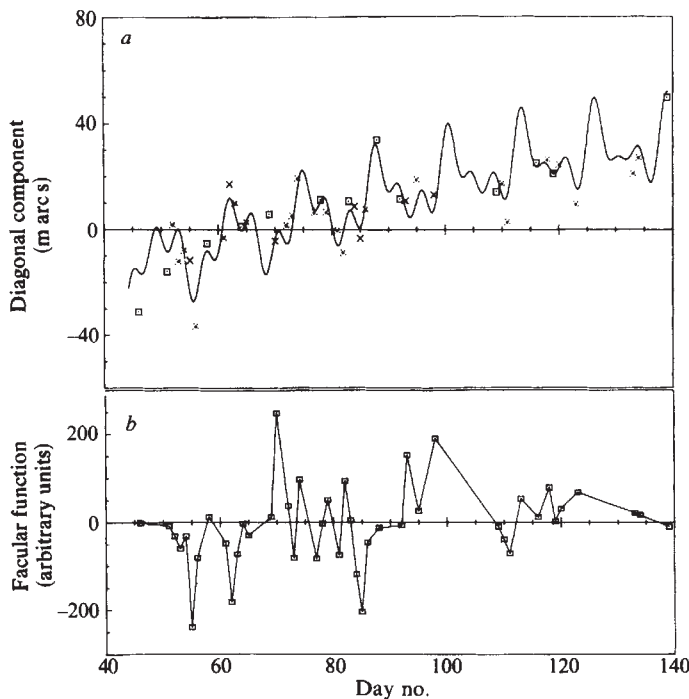


Fig. 1 *a*, The diagonal component of the solar ellipticity corrected for instrumental errors (that is mirror distortions), atmospheric refraction and facular signals ($-c_t F_c$). $\square$, Weak facular signals, $|F_c| < 14$. $\times$, Strong signal, $|F_c| > 100$. $\times$, $14 < |F_c| < 100$. *b*, The facular function F_c plotted for days with data in *a*. The function F_c fits the data points very poorly. The data points have been corrected for the presence of the facular signal by subtracting the computed facular signal. The facular signal subtracted is $0.068 F_c$, where F_c is obtained from curve *b*.

During the summer of 1966 the Sun was quiet and for 20–30% of the observational days there were only a few weak facular patches at the solar limb. But the ‘ellipticity signal’ did not disappear on those days⁴, hence cannot be wholly facular in origin, a conclusion which is independent of any assumption concerning the facular contrast function. To avoid subjectivity we use Chapman’s facular function F_c to select the days with negligible facular signals instead of photographs or maps³.

In Fig. 1 the data for the ‘diagonal component’ of the solar ellipticity² are plotted for the days selected in this manner. The curve is the calculated diagonal component of the ellipticity associated with the 12.4-day rotating distortion. (See ref. 8 for the treatment of the data and the calculation of the curve.)

Schatten and Sofia express the excess facular light flux from an annulus at the solar limb as a sum over the area of the facular patches in the annulus multiplied by $f(\mu)$ where f (our notation) is the product of three terms: a foreshortening factor μ , a limb-darkening factor and the facular contrast function. Here $\mu = \cos \theta$ and θ is the angular distance from the centre of the solar disk.

Three different occulting disks were used in 1966, disk numbers 1, 2 and 3, with the edges of the occulting disks respectively

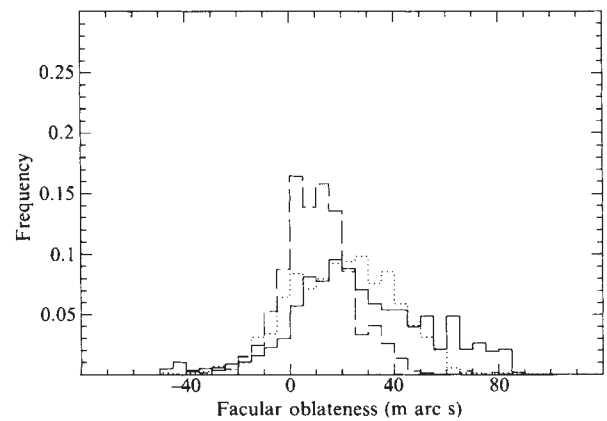


Fig. 2 Distribution of the facular contribution to the apparent solar oblateness obtained from 1,600 observations taken during 1982. Solid curve is for disk 1, dotted curve is for disk 2, and the dashed curve shows the distribution from disk 3 data.

at $\theta = 79^\circ, 81.1^\circ$ and 84° . For faculae randomly positioned on the Sun’s surface but falling in annulus number 1, the expectation value of $f(\mu)$ is $\langle f(\mu) \rangle_1 = \int_{\theta_1}^{\theta_2} f(\mu) d\theta / 11^\circ$, where $11^\circ = 90^\circ - \theta_1$. For the faculae to fall also in annuli 2 or 3 the limit θ_2 or θ_3 is substituted in the integral to obtain the expectation value for $f(\mu)$. (The denominator 11° is not changed.) For a sufficiently large number of faculae, irregularly positioned in this way, $f(\mu)$ can be replaced by its expectation value for each annulus. The facular signals are then calculated from the facular area contained in annulus number 1 multiplied by the three expectation values $\langle f \rangle$. The areas of facular patches contained in annulus number 1 were measured by Chapman³ for the 1966 data. The expected facular contribution to the diagonal component of the ellipticity is plotted as F_c in Fig. 1.

The expectation values of $f(\mu)$ are given in Table 1 for the three different limb exposures and for three different assumed facular contrast functions (the lower three curves at the right of Fig. 1 of ref. 1). To compare with the ellipticity measurements, the expectation values $\langle f(\mu) \rangle$ in Table 1 are divided by I_e , the relative brightness of the Sun at the edge of the occulting disk.

To correct for facular contamination of the data the term $c_t F_c$ is added to the ellipticity function in least-square fits to the data^{2,8}. The three values of c_t obtained in this manner⁸ are given in Table 1. These three values should be proportional to $\langle f(\mu) \rangle / I_e$ for an acceptable contrast function. The best agreement is with the third contrast function, a constant contrast, but the Schatten and Sofia function is almost as good.

Note that individual faculae in a facular patch are correlated in position and the resulting error in using the expectation value for $f(\mu)$ can be appreciable. However, the time average over an observational day improves this approximation. The window used by Chapman in computing F_c does not coincide perfectly with θ_1 , that is with the annulus number 1.

Measurements obtained during the summer of 1982⁹ are consistent with this determination of the facular contribution to the 1966 solar distortion measurements. Direct photoelectric

Table 1 Expectation values of $f(\mu)$

Disk no.	Exposed limb* (arc s)	$\theta^\dagger$ (deg)	$\mu^\ddagger$	$I_e^\ddagger$	$\langle f_1(\mu) \rangle / I_e^\S$	$\langle f_2(\mu) \rangle / I_e^\S$	$\langle f_3(\mu) \rangle / I_e^\S$	c_{tl}
1	17.9	79.0	0.192	1.00	0.0125	0.0057	0.0029	0.101 ± 0.011
2	11.6	81.1	0.155	0.95	0.0104	0.0039	0.0019	0.061 ± 0.011
3	5.3	84.0	0.100	0.84	0.0076	0.0020	0.0009	0.016 ± 0.016

* Annular width.

† Position of edge of the occulting disk.

‡ Relative brightness at edge of occulting disk.

§ The expectation values of $f(\mu)$ are computed for: (1) Chapman’s function, (2) Schatten and Sofia function, (3) constant (see text).

$||$ See ref. 8.

Table 2 Facular oblateness contribution

Disk no.	Exposed limb* (arc s)	Mean 1982 faculae† (m arc s)	Extrapolated 1982 faculae‡ (m arc s)	Relative extrapolated 1982§	Relative 1966
1	20.8	26.5 ± 1.1	8.4 ± 0.3	1.00 ± 0.04	1.0 ± 0.1
2	14.5	20.3 ± 0.7	6.0 ± 0.2	0.71 ± 0.03	0.6 ± 0.1
3	8.0	10.4 ± 0.5	2.5 ± 0.1	0.20 ± 0.02	0.2 ± 0.2

* Annular width.

† Mean facular contribution to the oblateness, $\Delta r_{eq} - \Delta r_{pole}$.

‡ 1982 facular contribution extrapolated to 1966 (see text) from 1982 data (see text).

§ Column 4 renormalized.

|| c_t from Table 1, renormalized.

observations of the facular contribution to the integrated flux from the limb yield a contrast function which is inconsistent with the Chapman and Klabunde⁷ result.

The 1982 observations allow a direct measurement of the facular contribution to the inferred solar ellipticity. Small-scale facular contributions to the integrated light flux can be separated from the ellipticity signal because the data were obtained with an angular resolution of $\sim 3^\circ$ around the limb. The frequency distribution of the facular contribution to the oblateness for three different limb exposures is shown in Fig. 2. These were obtained from $\sim 1,600$ observations collected during 1982⁹. Figure 2 clearly shows the decreasing importance of the facular contribution to the oblateness signal as the limb exposure is reduced. This variation of facular signal with limb exposure is shown quantitatively in the third column of Table 2, the mean facular contribution to the oblateness signal.

The ratios of these three facular oblateness signals cannot be directly compared with the ratios of c_t of Table 1, for the limb exposures were rather different in 1982. After correcting for the differences in limb exposure and also correcting for the reduced solar activity in 1966 and the change in the dominant latitude of solar activity, we obtain the extrapolated values given in column 4 of Table 2. These extrapolated values provide our best estimates of the relative contributions of facular signals to the apparent solar oblateness in 1966 for disks 1, 2 and 3, based on the 1982 observations. These three values should be proportional to the three values of c_t in Table 1, as indeed they are (see columns 5 and 6 of Table 2). Column 4 also provides rough estimates of the magnitudes of the facular contribution to the uncorrected oblateness signal in 1966. Due to the low solar activity in 1966 and the resulting large fluctuation in F_c , these estimates may be reliable to only $\pm 50\%$. Comparing columns 3 and 4 of Table 2, the facular signals for 1982 are seen to be substantially larger than the signals for 1966 estimated from the 1982 observations.

Figure 1 shows that F_c is not well described by the time dependence of the diagonal component of the solar oblateness. Furthermore, it is the large difference between the two curves in Fig. 1 that allows least-squares evaluation of c_t and the subtraction of the facular signal from the 1966 data.

The recent facular contrast measurements⁹ and the direct determination of the facular contribution to the limb distortion signal support our claim that the 1966 solar ellipticity measurements were not seriously contaminated by facular brightness. This is consistent with the conclusion of Schatten and Sofia assuming the correctness of their facular contrast function.

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15-Myr periodicity in the frequency of geomagnetic reversals since 100 Myr

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The statistical properties of the magnetic polarity time scale have been examined recently^{1,2} for several time scales. For some of these scales the time evolution of the frequency of reversals as seen through an 8-Myr moving rectangular window have been determined. In particular, over the past 100 Myr, the mean reversal frequency appears to be dominated by a linearly increasing trend on which is superimposed a rhythmic fluctuation. We have attempted to analyse the mathematical structure of the reversal frequency curve in detail and to determine whether the rhythmic fluctuation was indeed periodic. By modelling the time evolution of the reversal frequency, we find a dominant frequency corresponding to a period of ~ 15 Myr. This result represents one of the most persistent long-term periodicities ever reported for a geophysical phenomenon.

Our study of the frequency of reversals is based on two different time scales, each of which covers the past 100 Myr. The first, LA, is the time scale of Lowrie and Alvarez³ which contains 174 reversals. The second, LKC, is that of Labreque *et al.*⁴; this time scale differs slightly from LA in the assignment of some geological ages and it also includes some short polarity reversals, mainly in the interval between 8 and 12 Myr. Each of these reversals has an estimated duration of $< 40,000$ yr, but the existence of each one is supported by the presence of characteristic and coherent features in marine magnetic anomaly profiles^{5,6}. The LKC time scale contains 191 reversals. Both these and previous scales have also been studied by Lowrie and Kent².

The frequency of reversals as a function of time for each time scale was calculated from original data using the normal technique of a moving rectangular window^{1,2,7}. Window widths ranging from 2 to 8 Myr were examined. The step interval depended on the window width, but in all cases the interval was < 0.1 Myr. The results for the two time scales using a window of 4 Myr are shown in Fig. 1.

The reversal frequency curves were then analysed in terms of a monotonically-increasing component and an oscillating component. The monotonic component was modelled by a least-squares fit for two different functions, a lorentzian and a straight line. (The formula for the lorentzian function, which is also known as the Cauchy function, is $2\sigma / ((t - t_0)^2 + \sigma^2)$.) The

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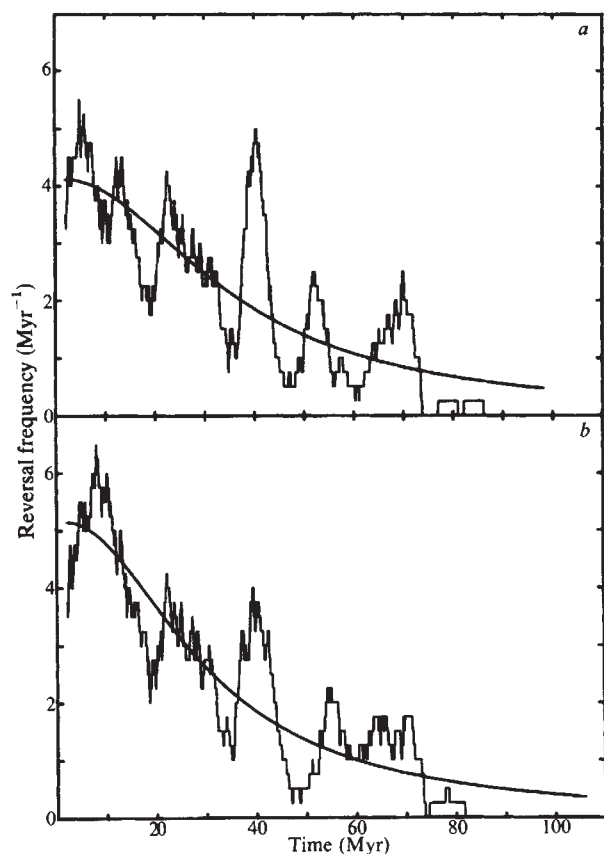


Fig. 1 Frequency of reversals as seen through a moving window with a width of 4 Myr. *a*, LA time scale; *b*, LKC time scale. The smooth curves are lorentzian functions, where σ and t_0 were determined by a least-squares fit.

lorentzian curves for the 4-Myr window are shown in Fig. 1. For time scales LA and LKC, respectively, half-widths (σ) of 35 and 29 Myr were obtained. Within the accuracy of the analysis, the location of the peak (t_0) of the lorentzian functions was not significantly different from 0 Myr. Therefore in subsequent analyses, the peak was fixed at 0 Myr.

To analyse the oscillating part of reversal frequency curve, we subtracted the corresponding lorentzian from its reversal frequency curve and then performed a time autocorrelation on the remainder. Figure 2 shows the results for the 4-Myr window. It is remarkable that both curves show a periodicity corresponding to a period of ~ 15 Myr. The periodicity is clearer and more distinct in LKC due to the effect of the short reversals on the reversal frequency between 8 and 12 Myr. Because the evidence for these short reversals is strong⁸, we believe that LKC is probably a better representation of the time scale during this interval. The same periodicity was also found when windows ranging from 2 to 8 Myr were used, however, a window width of 4 Myr represented the best compromise between the noise arising from the discrete nature of reversals and the need to obtain adequate resolution of the reversal frequency.

Based on the foregoing analysis, we proceeded to fit the reversal frequency curve of LKC simultaneously with a lorentzian function and a sine function. For the 4-Myr window, this produced a half-width of 31 Myr for the lorentzian and a period of 15.1 Myr for the sine function (Fig. 3). The same approach using a straight line and a sine function gave a period which differed by only 2%; however, it represented a less satisfactory overall fit.

It is unlikely that the monotonically increasing function represents an observational effect resulting from the greater likelihood of observing young reversals. The principal sources of data for the magnetic polarity time scales beyond ~ 10 Myr are marine magnetic anomalies and sequences of exposed marine

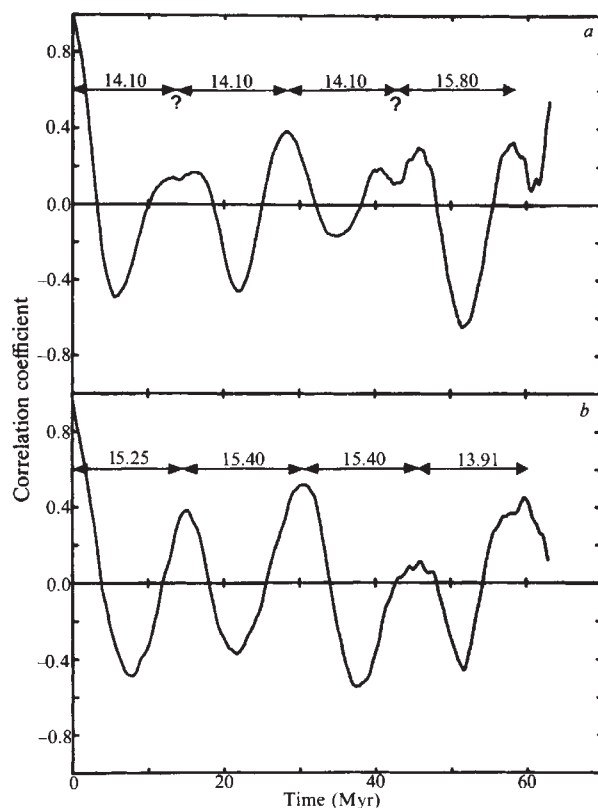


Fig. 2 Time autocorrelation of the curves shown in Fig. 1 after subtraction of the lorentzian component. *a*, LA time scale; *b*, LKC time scale.

sediments. The presence of polarity intervals with durations $< 250,000$ yr in marine magnetic anomalies of all ages, and the fact that these features can be correlated globally, are evidence that the polarity record in marine magnetic anomalies has not significantly degraded with time. In addition, some of the most detailed magnetostratigraphical studies have focused on late Cretaceous and early Palaeogene marine sequences⁹ so that an observational effect would manifest itself as a peak in reversal frequency for this time range, which is not seen.

Thus, we believe that the geomagnetic reversal frequency can be analysed in terms of a monotonically increasing function and an oscillating function. Our evidence does not constitute proof that the corresponding physical processes actually do exist in the Earth's core, and, indeed, we cannot suggest any physical mechanism which would produce the observed mathematical structure. Our result allows for considerable flexibility

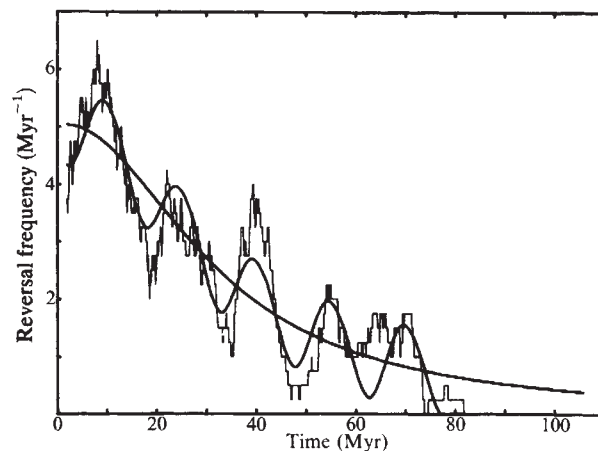


Fig. 3 Direct least-square fit of the LKC time scale using a lorentzian function plus a sine function.

in the development of an appropriate theory. For example, the monotonically increasing component may represent an actual long-term process within the core that serves to control the overall frequency of reversals. However, the exact shape of the function is open to additional analysis. On the one hand, the lorentzian function gives a very satisfactory fit to the reversal frequency curve, and many physical processes involving relaxation and regeneration give rise to lorentzian line shapes. On the other hand, other plausible functions, such as an exponential or a tangent, could probably fit the data just as well, and there is no reason to assume that the present time represents a critical point in geomagnetic history, as is implied by the location of the peak of the lorentzian curve. The monotonically increasing function may itself be part of a fluctuation in the reversal frequency with a period of 350 Myr, as has been previously proposed¹⁰.

With regard to the oscillating component, we have modelled it as a process which serves both to increase and decrease the

reversal frequency above and below that determined by the monotonic function. The oscillating component can also be interpreted as a perturbation which only increases or only decreases the reversal frequency above or below a level determined by monotonically increasing functions with different parameters. Within the context of these interpretations, different values of the monotonically increasing function would make the field either more or less stable towards reversal, a phenomenon which mathematically, at least, can be modelled by the theory of chaotic dynamics¹¹. All of these possibilities must be considered when searching for a physical interpretation of this component. However, regardless of its origin, a periodicity of 15 Myr continuing over an interval of 80 Myr represents one of the most persistent periodic, long-term geophysical phenomenon ever reported.

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Two different R–N geomagnetic reversals with identical VGP paths recorded at the same site

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We have studied two reverse–normal (R–N) geomagnetic reversals recorded at different stratigraphical levels of a single section of Tortonian marine clays in western Crete. We report here that the virtual geomagnetic pole (VGP) paths associated with these two transitions, which are separated in time by a period of ~1 Myr, are identical. Although the possibility of an accidental coincidence cannot be rejected, this result suggests that the basic mechanisms in the geodynamo leading to a reversal might persist unchanged over a long period of time. Moreover, the two VGP paths are largely constrained in longitude along a mean great circle located ~80°W of the site, so that the transitional field cannot be correctly described in terms of pure axial symmetry.

Palaeomagnetic studies of the reversals of the geomagnetic field have provided new ideas and data about the mechanisms and structure of the Earth's dynamo. Surprisingly, however, no attention has been paid to a possible long-term persistence of the structure of the transitional field. We now consider two R–N transitions recorded in a section of very fine-grained Tortonian marine clays with a total thickness of 54 m, near the village of Skouloudhiana in western Crete. Previous magnetostratigraphical work¹ has established the presence of six successive polarity reversal horizons in this section: the two studied in this work are the second (KS02) and the sixth (KS06) ones from the bottom of the section. They are separated by a stratigraphical distance of 42 m. The four additional polarity transitions present in the section have not been studied because the NMR intensity is very low at the corresponding horizons, so that the transitional samples could not be measured with the required accuracy.

Sampling was done using standard palaeomagnetic tech-

niques. Careful attention was paid to determining the exact relative stratigraphical position of the cores in each transitional zone. Repetitive measurements showed that the location of each sample could be determined with a precision of 4 mm.

Although the section between KS02 and KS06 contains an 8-m unexposed interval, its continuity is well established by the previous magnetostratigraphical and biostratigraphical results. In particular, the two transitional zones belong to two different biozones¹. Moreover, the stratigraphical intervals embraced by the two transition zones are significantly different and the clays from these two zones contain different amounts of magnetic minerals: the average low field magnetic susceptibilities of ~20 samples from each of the two zones differ by a factor of two and their saturation isothermal remanent magnetization by a factor of four. Thus it is very unlikely that a large fault, passing exactly through the unexposed portion of the section, would cause the same reversal to appear twice in the sequence. In addition, no indication of such a fault was found in the field.

Many sedimentary and mineralogical properties of the section were also investigated. In particular, the anisotropy of the magnetic susceptibility of the clays has been measured at different horizons of the section. The results show that the magnetic fabric of the Skouloudhiana section is characterized

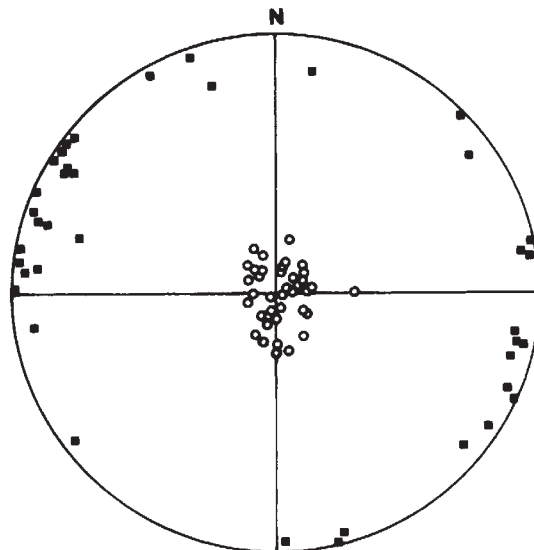
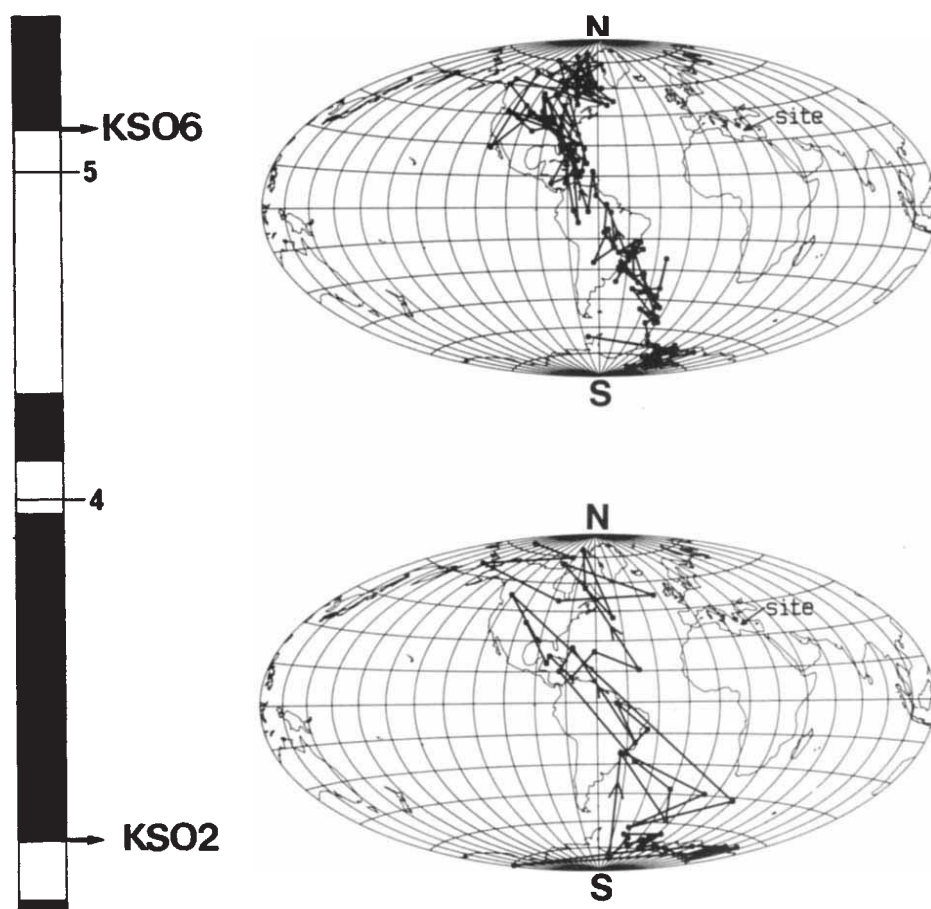


Fig. 1 Stereographic projection of the axes of minimum (○) and maximum (■) magnetic susceptibilities of samples from different horizons of the entire Skouloudhiana section.

Fig. 2 VGP paths of the two polarity transitions plotted in the planisphere centred at 300° E long. (80° W of the site meridian). The magnetostratigraphical column on the left of the figure gives the position of the two reversals in the section. 5, Entry of *G. Menardii* form 5; 4, exit of *G. Menardii* form 4.



by an oblate susceptibility ellipsoid with a very moderate (1.05) degree of anisotropy². The mean axis of minimum susceptibility, $K_{\min}$, is perpendicular to the bedding plane ($\bar{I} = 88.5^\circ$) while the axes of maximum susceptibilities of the individual samples, $K_{\max}$, are very largely scattered in direction in this plane (Fig. 1). This indicates a planar sedimentary fabric. These results thus provide good evidence of a calm water and subsequently unperturbed deposition of the sediments. Distortion of the results by bottom current or by tectonic stresses, if any, is certainly very limited.

A total of 57 samples for KS02, and 195 samples for KS06 were stepwise thermally demagnetized. The final palaeomagnetic directions were determined from the demagnetization diagrams. Except for a small viscous component removed at low temperatures, only a single stable component of magnetization was found up to the highest temperatures. The final palaeomagnetic directions were thus determined with good accuracy in most samples (only 10 out of 252 samples did not yield a stable direction). However, a larger scatter in the directions was observed in the case of the lowest magnetized samples, even though a cryogenic magnetometer was used for these measurements. This was the case for the samples of the KS02 polarity transition, all of which are in a weakly magnetized zone, and this explains the larger scatter observed in the data of this transition. Despite this scatter, both records are largely classifiable in 'category A', as defined by Fuller *et al.*³. In particular, the large number of intermediate directions makes KS06 the most detailed reversal studied so far, and a detailed account will be published elsewhere.

From the directions obtained after demagnetization, the successive VGP positions have been calculated for the two reversals. The results are shown in Fig. 2, together with a stratigraphical column giving the position of the two reversals in the section.

The two paths are largely constrained along two mean longitudes which differ by $\sim 30^\circ$ in the Southern and Northern

Hemispheres. Accurate determinations of pole positions from many samples well outside the transition zones, however, also show this same asymmetry. Thus, this unexplained feature is related to properties of this particular section and not to the reversal mechanism.

The main feature shown in Fig. 2 is that, within noise limits, the VGP paths associated with the two polarity transitions are identical. This same VGP path has also been observed for a R-N transition recorded in a nearby section at Potamida⁴, which, according to magnetostratigraphical work, is the same reversal as KS06.

The coincidence of the Skouloudhiana paths appears, at first sight, to be consistent with the standing-field model of geomagnetic reversals⁵. However, the N-R transition which immediately follows in time and is recorded in Potamida, has a quite distinct VGP path $\sim 135^\circ$ away from that of the R-N transition⁵. Therefore, the coincidence in the VGP path is limited to the two R-N transitions, and does not appear in the N-R transition as required by the standing-field model.

The R-N paths recorded at Skouloudhiana are, on average, $\sim 80^\circ$ long. away from the site. Other reported paths from R-N transitions are either closer to the sites or, as in the case of a transition described by Williams and Fuller⁶, $\sim 120^\circ$ away from the site. Thus, the results from Skouloudhiana confirm that the transitions cannot be as simply classified as 'near' or 'far sided' as originally suggested by Hoffman⁷. On the contrary, as stated in ref. 6, one should not postulate that all the geomagnetic reversals have the same transitional geometry. Thus the coincidence in the VGP paths of the two different transitions in Skouloudhiana is particularly interesting.

Similarities, or even coincidences, among VGP paths of different transitions recorded in different but geographically close sites have already been noted by others⁸⁻¹⁰. In some cases, however, such as the R-N transitions recorded in the Tatoosh intrusion at Mt Rainier¹¹ and in the Laurel Hill intrusion at Mt Hood¹², the records show considerable scatter and must be

heavily smoothed before comparison^{9,10}. In other cases, such as the N-R transition recorded at Searles Valley and the R-N transition at Lake Tecopa⁸, the coincidence is striking but as the author himself points out, some uncertainty exists because the N-R transition was recovered from a rotary-drilled core and an assumption has been made about absolute declination. Thus we believe that the present result is the clearest reported example of identical VGP paths from two different transitions of the same sense recorded at the same site. Although this coincidence could be purely accidental, there are other, possibly more realistic hypotheses. For example, some conditions in the core may favour a particular reversal mechanism, so that reversals of the same sense will always occur with a definite configuration as long as these conditions prevail. This situation would cause, in particular, all the successive reversals of the same sense recorded at the same site to have identical VGP paths over some period of time, which, according to the present results, would be at least 1.2 Myr.

Alternatively, only a very limited number of possible reversal configurations might be liable to occur with finite probability at any epoch, so that only particular ones will be observed rather frequently in time. This possibility has already been considered by Hoffman in connection with the Laurel Hill and Tatoosh reversals⁹. This interpretation does not imply that successive reversals of the same sense all necessarily have the same transitional field. Although the presently available results

do not yield a clear-cut answer, we believe they render the first hypothesis more realistic. Definite conclusions will presumably only be reached when results from long-time sequences obtained at the same site become available.

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Palaeomagnetic evidence for early Pleistocene in the central and northern North Sea

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The palaeomagnetic evidence presented here refutes the earlier hypothesis that most of the Quaternary succession in the central North Sea area is of Middle to Upper Weichselian age. A study of the Quaternary seismic-stratigraphy has been tied to 11 cored boreholes which are being examined for their physical properties and faunal content. Initial palaeomagnetic studies show a major reversely magnetized zone which can be correlated from borehole to borehole and with the seismic stratigraphy. This reversely magnetized zone is thought to represent the Matuyama Epoch which ended ~790,000 yr BP; consequently this suggests the presence of early Pleistocene strata in the central and northern North Sea.

Good quality seismic profiles have been run by the Institute of Geological Sciences (IGS) throughout the North Sea area, and have been interpreted by the Marine Geology Unit of IGS for 1:250,000 map compilations. Using these data, a seismic-stratigraphical sequence has been erected, with individual seismic units being defined by prominent seismic reflectors. Many of these seismic reflectors can be correlated over large areas and represent lithological variations, geotechnical changes, erosional unconformities and/or non-sequences. Erosional unconformities are recognized as irregular channelled surfaces. In the central and northern North Sea area a prominent seismic reflector is interpreted as a major erosion surface, and it is penetrated by all the boreholes described here (Figs 1, 2). In the central North Sea the sediments below this erosional discontinuity have been termed the Aberdeen Ground Beds¹.

Despite the well established seismostratigraphical framework for the Quaternary, chronostratigraphical subdivision of the sequence has remained uncertain. Several workers^{1,2} have suggested on the basis of radiocarbon dates that the bulk

of the Quaternary sequence (including the Aberdeen Ground Beds) is of Middle to Upper Weichselian age. These sediments are separated by a marked crenulate reflector from a basal unit of unspecified early Pleistocene age. This unit has been identified only on digitally-processed sparker records which are not available over much of the study area. This unit is therefore not shown on Fig. 2.

Repeated major erosion during the glacial stages was suggested as an explanation for the apparent lack of a significant thickness of pre-Weichselian sediment³. In contrast, other workers⁴⁻⁶ have presented alternative seismostratigraphical interpretations and palaeoclimatological evidence to suggest that a more complete Pleistocene succession may be present, hence casting doubt on the reliability of the radiocarbon dates mentioned above.

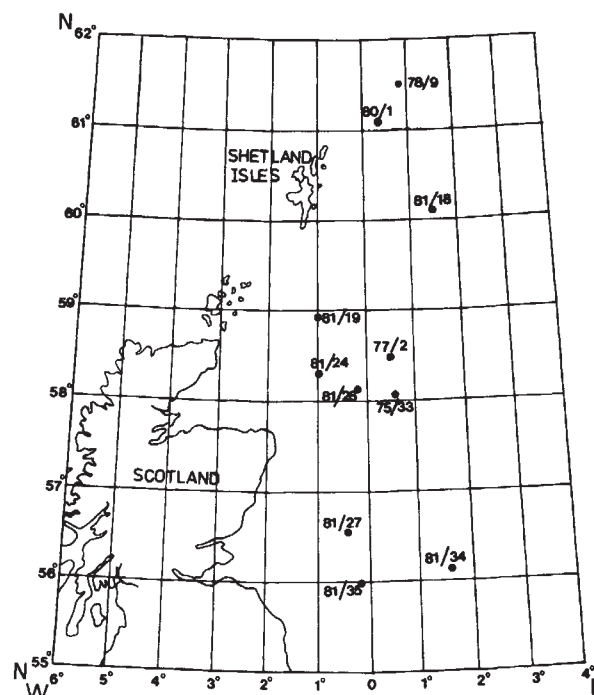


Fig. 1 Location of IGS boreholes. All boreholes were rotary cored from seabed for their entire length.

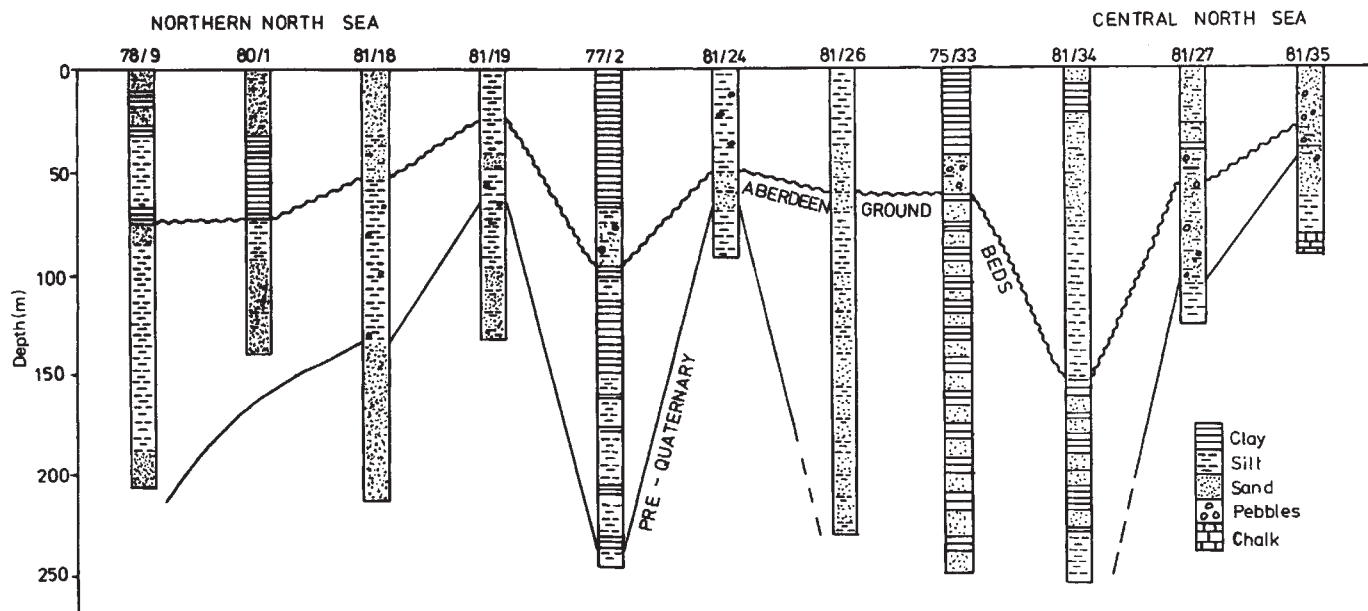


Fig. 2 Correlation of IGS boreholes using seismic stratigraphy derived from IGS seismic profiles. The correlation lines shown indicate major seismic reflectors which are visible on records over the entire area of survey.

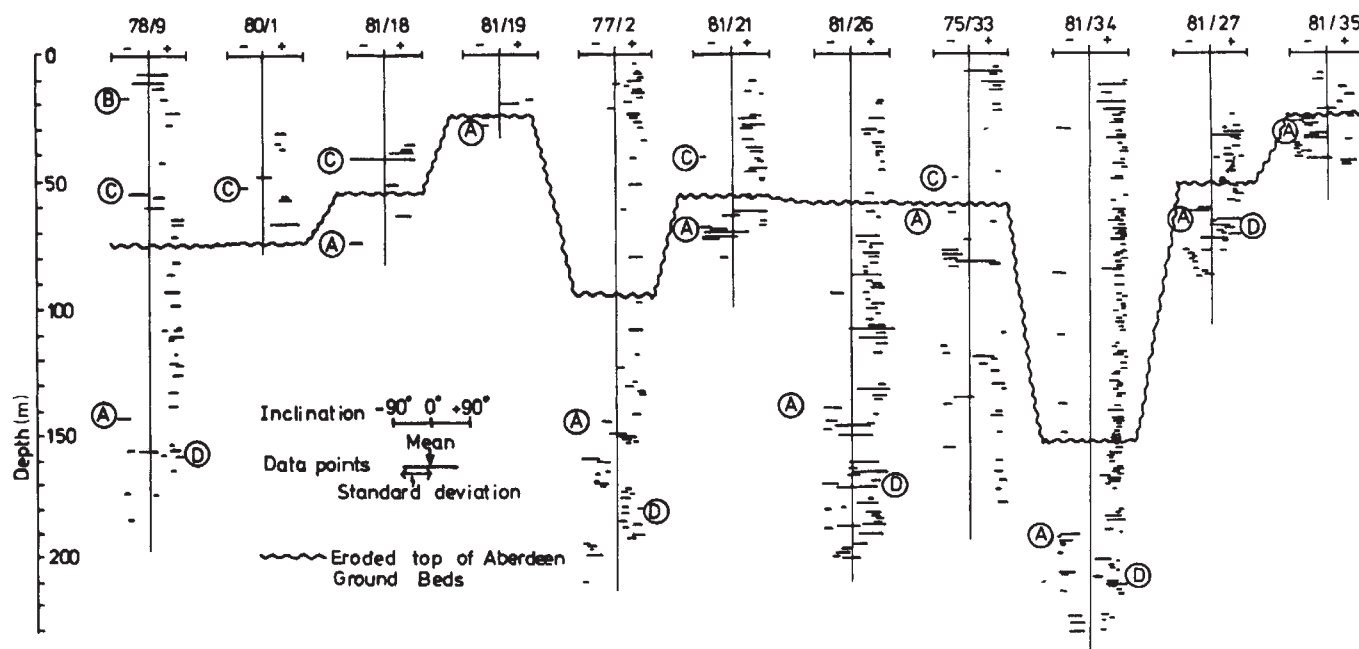


Fig. 3 Correlation of the palaeomagnetic results. The Brunhes-Matuyama boundary (~ 790 kyr BP) is denoted by A; B, the Laschamp and Olby excursion (~ 36 – 42 kyr BP); C, Blake event (~ 108 – 114 or 104 – 117 kyr BP); D, Jaramillo event (~ 890 – 950 kyr BP).

The present study was prompted by the results of an earlier investigation by one of us (A.C.S.) which demonstrated normal and reversely magnetized zones in IGS borehole 78/9, and which were subsequently correlated in detail with the palaeoclimatic succession derived from micropalaeontology⁷. It is hoped that further work on the same borehole using oxygen isotope techniques will be published shortly. Using this information palaeomagnetic measurements were carried out on 10 other borehole sequences in an attempt to make regional lithostratigraphical and chronostratigraphical correlations, and to obtain a reliable framework on which to build up the Quaternary stratigraphy of the central and northern North Sea.

The boreholes were sited to provide controls for the seismic stratigraphy. Most are located in areas of uniform reflectors to obtain the general stratigraphy, but some have been situated on stratigraphical highs or over buried channels with a view to providing more detailed information on the nature of those features present. Pre-Quaternary strata were also cored in some

of the boreholes to obtain a thickness for the Quaternary sequence and date the underlying strata. In all boreholes the predominant lithology is interbedded clays, silts and sands. Shells, shell fragments and pebbles are also present in places, but rarely were typical 'till-like' deposits encountered, and most of the sediments are interpreted as glaciomarine or marine deposits.

Samples for palaeomagnetic study were taken, where possible, at 1-m intervals throughout the core. A minimum of three samples from each horizon ensured sample orientation reliability. Each sample was measured for natural remanent magnetism (NRM) using Digico and cryogenic magnetometers. Demagnetization in fields of 5, 10, 20, 30, 40 and 60 mT was carried out on selected samples close to 0° inclination in four of the cores (75/33, 77/2, 81/24, 81/26), and it was noted that the results did not materially affect the polarity pattern obtained from the NRM results. Consequently only the NRM was used when constructing Fig. 3 which is intended for regional

correlation only. Note also that no curves are drawn between sample points in the sections as seismic interpretation reveals that discontinuities exist within the cores.

The polarity results from the longer boreholes can be subdivided into two main zones, an upper zone of dominantly normal polarity and a lower zone of mainly reversed polarity. By comparing our results with published data regarding the geomagnetic reversal time scale⁸⁻¹¹, we suggest that the upper zone represents the Brunhes Normal Epoch and the lower zone represents the Matuyama Reversed Epoch. The Brunhes-Matuyama boundary (A on Fig. 3) is currently taken at $\sim 790,000 \pm 5,000$ yr BP (ref. 12) and a detailed study of this boundary showed that the reversal was completed in $< 1,000$ yr (ref. 13). This simplified division is complicated by the occurrence of several anomalous short-geomagnetic transitions. It is debatable whether these kind of features in reality reflect a global change of the Earth's magnetic field. In the Brunhes Epoch the occurrence of certain anomalous reversed zones is well documented. These reversed events and/or excursions include the Chagan or Biwa 1¹⁴ (292,000–298,000 yr BP or 260,000–280,000 yr BP), Biwa 2¹⁴ (176,000–186,000 yr BP), Blake¹⁴ (108,000–114,000 yr BP or 104,000–117,000 yr BP), and Laschamp and Olby^{15,16} (36,000–42,000 yr BP). The possible position of the Blake event and Laschamp/Olby excursion has been indicated, where possible, on Fig. 3, this suggested location having been supplemented by unpublished micropalaeontological and oxygen isotope data from the relevant boreholes. Supplementary data to assist in assigning any names to the other reversely magnetized zones within the Brunhes Epoch is currently lacking, hence some of the anomalous zones illustrated on Fig. 3 remain uncorrelated at present.

The Matuyama Reversed Epoch is characterized by two well-documented normal events within the Quaternary sequence, the Jaramillo and Olduvai events^{8,9,11}. We have indicated on Fig. 3 the possible location of the Jaramillo event ($\sim 890,000$ – $950,000$ yr BP) in our cores although there are little supplementary data to confirm this. In addition, at least two further normal events have been recorded in a sequence of sediments at Boso in Japan occurring between the Jaramillo and Olduvai events¹⁷. Moreover, polarity data from continental North-west Europe¹⁸ also indicate several normal events or excursions within the dominantly reversed Matuyama Epoch. Hence our data appear consistent with published material.

The recognition of the Brunhes-Matuyama reversal is of considerable importance in the erection of a Quaternary chronostratigraphy for the central and northern North Sea. The new data, if correct, suggest that the Quaternary succession in the North Sea represents a more complete sequence of Pleistocene sediments than has previously been suggested¹⁻³. Where the sequence is not attenuated the Brunhes-Matuyama boundary occurs within the Aberdeen Ground Beds, below the erosion surface which marks the top of this unit, implying a Menapian/Cromerian (Complex) age for this part of the succession. Below top Matuyama preliminary micropalaeontological (R. Harland, personal communication) and macro-palaeontological (D. K. Graham, personal communication) results support an interpreted early Pleistocene age of the Aberdeen Ground Beds, in some instance as old as the Tiglian. Higher up within the stratigraphical sequence the identification of Eemian strata in borehole 78/9 (~ 50 m)⁷ suggests, by inference, that the major erosion surface which truncates the Aberdeen Ground Beds is probably Saalian or Elsterian in age, and this is consistent with recent palaeoclimatological evidence regarding the age of this erosion surface⁶.

In conclusion the palaeomagnetic data have provided, for the first time, a regional Quaternary chronostratigraphical framework for the central and northern North Sea area, and have revealed a much more extensive Pleistocene sequence than was previously known. Unfortunately, over a long time span the shelf Quaternary sequence, with its significant erosion and non-depositional phases, is less complete than the oceanic record. However, the higher sedimentation rate on the shelf

gives a greater preservation potential for some of the shorter time range magnetic events or excursions which are often lacking in the oceanic record. Therefore, the new data allow for considerable advances to be made in the correlation and palaeogeographical reconstruction of the Quaternary of the North Sea.

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Note added in proof: Since submission of this letter, further micropalaeontological data have indicated the presence of two separate interglacial periods above the major erosion surface. This suggests that the erosion surface is of Elsterian age.

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Bowers graben and associated tectonic features cross northern Victoria Land, Antarctica

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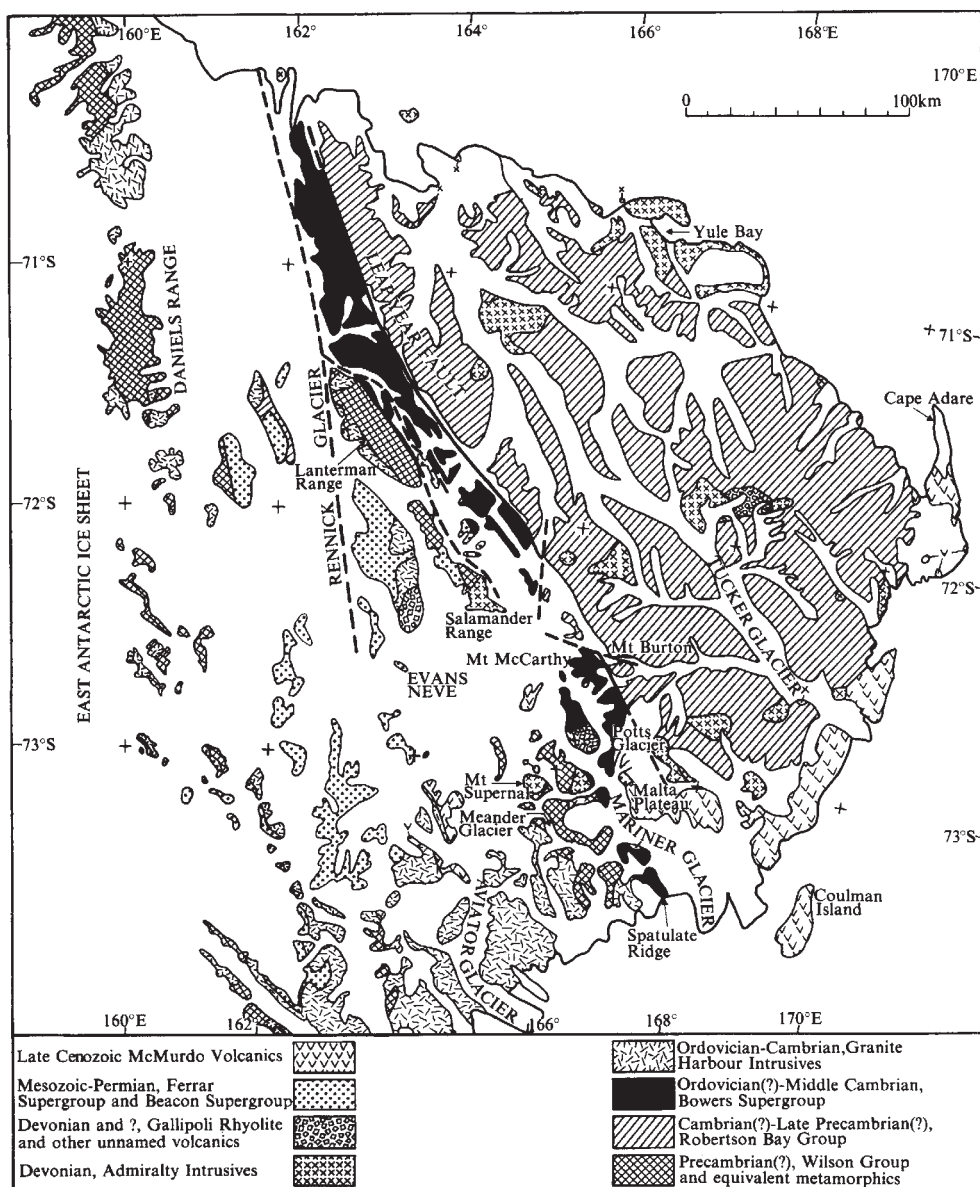
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Field mapping during the 1981–82 season in northern Victoria Land, Antarctica, has revealed that the Bowers graben, an elongate feature infilled with sediments of Middle to Upper Cambrian or Lower Ordovician age, extends entirely across northern Victoria Land from the northern coast to the mouth of Mariner Glacier (Fig. 1). The Leap Year Fault, postulated to be the eastern boundary of the graben has been confirmed this season in outcrop. Areas west of the Bowers graben, previously mapped as Robertson Bay Group south-west of Mariner Glacier, are in fact equivalents of Wilson Group which occurs farther north along regional structural trends. Early and middle Palaeozoic granitoids appear divisible into S- and I-types with the Bowers graben marking the approximate eastern limit of S-types. We show here that the Bowers graben and associated tectonic features mark a major crustal boundary. Because the graben strikes south-east into the Ross Sea without emerging elsewhere in the Transantarctic Mountains, the rocks of the graben and those east of it represent a geological terrain which appears to be unique throughout the Transantarctic Mountains. This boundary zone is of fundamental importance in the tectonic architecture of the region and is an important consideration in any reconstruction of Australia and Tasmania with the Antarctic continent^{1,2}.

Fig. 1 Geological map, northern Victoria Land, Antarctica. Portions adapted from refs 19, 32, 33.



Previous workers had established the presence of a narrow trough, 20–25 km wide, extending from the northern coast southwards to upper Mariner Glacier, which contains a lithologically-variable suite of rocks called the Bowers Supergroup^{2–14}. Sedimentological analysis has suggested that these rocks were deposited in an elongate, structurally controlled, trough-like basin of Middle to Upper Cambrian or Lower Ordovician age¹¹.

To the west in the Lanterman Range, the Bowers Supergroup is bounded by metamorphics assigned to the Wilson Group^{4–6,12–14}, along a complex steeply-dipping fault zone within which an unconformable contact is preserved in some fault slivers. The metamorphic aspect of the Wilson Group has led workers to suggest a Precambrian age, although this is unconfirmed by isotopic dating.

To the east the Bowers Supergroup is limited by the late Precambrian(?)–Cambrian(?) Robertson Bay Group, a widespread turbidite sequence which has been folded along north-westerly trending axes^{3–9,14–18}. The boundary is the Leap Year Fault, which until this field season had been inferred by the close proximity of younger units of Bowers Supergroup to Robertson Bay Group rocks and by increased deformation in the Robertson Bay Group along its western margin.

During the past season the Leap Year Fault was located in outcrop approximately midway along the ridge between Mt

Burton and Mt McCarthy. It is a highly sheared zone ~500 m in width with a near-vertical attitude on the shear planes. The fault extends southeastward where it is concealed beneath ice. The Leap Year Fault is now recognized as a major tectonic feature essentially continuous for more than 200 km from the Bowers Mountains to the Malta Plateau.

Helicopter reconnaissance mapping of numerous, previously-unvisited localities in the vicinity of Mariner and Meander Glaciers corrects the existing geological map which was based on photographic interpretation in the area¹⁹. Our mapping shows that Bowers Supergroup crops out along much of the length of lower Mariner Glacier. The most widespread unit is the Camp Ridge Quartzite, a brown to yellow sandstone and pebble conglomerate which has been mapped in the northern and central parts of the Bowers graben^{3–12}. The formation appears to coarsen to the south. It is particularly well exposed at Spatulate Ridge at the mouth of Mariner Glacier, where cross-bedded quartzose sandstone occurs with numerous horizons of pinkish, well-rounded quartz pebbles up to 3 cm in diameter. Slates of Mariner Group, probably Spurs Formation, crop out south of the mouth of Meander Glacier. These rocks are medium-grey, finely laminated, and contain sparse calcareous lenses. A few thin intercalation of quartzite also occur.

Dips on Bowers Supergroup are moderate to steep, with strikes generally along the trend of the graben. On the eastern

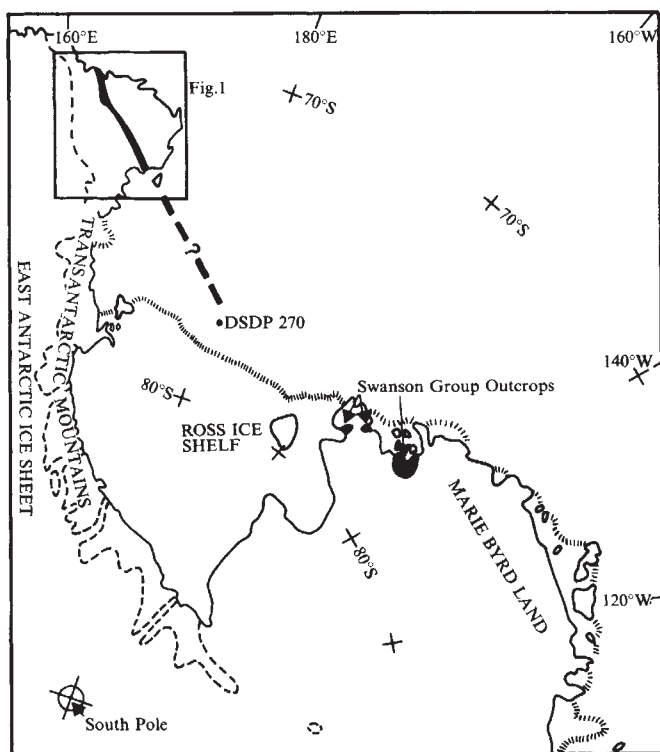


Fig. 2 Position of Bowers graben with respect to Transantarctic Mountains and Marie Byrd Land.

side of lower Mariner Glacier, lavas and hyaloclastites of the Cenozoic McMurdo Volcanics overlie Bowers Supergroup and the eastern boundary of the Bowers graben. Previously unmapped Beacon Supergroup rocks also crop out south of Potts Glacier (see Fig. 1).

To the west of the Bowers graben, in the vicinity of Meander Glacier, outcrops previously mapped as Robertson Bay Group¹⁹ proved to be a varied sequence of metamorphics assignable to the Precambrian(?) Wilson Group, as well as granitoid intrusions. At the southern end of Meander Glacier the rocks are highly flattened and the rocks are much more severely strained than those in the Bowers Supergroup. In the upper Meander Glacier area rock types include biotite schist with polyphase deformation and metavolcanics.

The presence of metavolcanics and clast-supported conglomerates argues against these rocks being metamorphic equivalents of Robertson Bay Group, which is, in general, alternating sandstone and shale with very minor matrix-supported conglomerate^{18,20}. The discovery of apparent Wilson

Group equivalents in the Meander Glacier area indicates that exposures along the entire western boundary of the Bowers graben, with the exception of younger igneous rocks, are of higher metamorphic grade and possible Precambrian age. The contact, however, has only been seen in the Lanterman Range^{21,22}.

Reconnaissance sampling of Lower and Middle Palaeozoic granitoid plutons throughout northern Victoria Land extends the observation made in the northern portion of the area that the Bowers graben marks the eastern limit of S-type granitoids²³. All known Devonian-age Admiralty Intrusives are I-type. They occur exclusively throughout the area east of the Bowers graben, and west of the graben are known only at Mt Supernal and the southern Salamander Range. Cambro-Ordovician-age Granite Harbour Intrusives are predominantly S-type, though some I-types also occur in the Lanterman Range and Daniels Range²³. These intrusives are found from the margin of the East Antarctic Ice Sheet to the Bowers graben, but not east of it, suggesting that the Bowers graben marks a major boundary in the lower continental crust. These preliminary statements are based on field observations, following mineralogical and inclusion criteria for I- and S-type granitoids²⁴.

The continuity across northern Victoria Land of the Bowers Supergroup, the Leap Year Fault, Precambrian(?) Wilson Group and the limit of S-type granitoids must be accounted for in any tectonic discussion of this region. Although the eastern limit of S-type granitoids may be carried into southeastern Australia where the I- and S-type classification was established²⁵⁻²⁷, in Australia the division of types occurs within granitoids of Devonian age, whereas in northern Victoria Land the Devonian granitoids are only I-type, and the S-types are Cambro-Ordovician in age. In Australia Cambro-Ordovician granitoids crop out west of Devonian occurrences in western Victoria and South Australia^{28,29}. This indicates spatial similarities and temporal differences in the crustal evolution of the two continents during the Lower to Middle Palaeozoic.

It has been suggested previously that the Bowers graben was linked to the Dundas Trough of Tasmania before continental breakup². To the south-east the Bowers graben strikes into the western Ross Sea (Fig. 2). The DSDP Site 270 is approximately along the projection of the graben. However, the basement rocks from the site are amphibolite-grade gneisses and schists, possibly correlative with metamorphics to the west, in the McMurdo Sound area³⁰. This suggests that the Bowers Graben, if continuous, passes north-east of DSDP 270. Previous workers have also suggested a correlation of the Robertson Bay Group with the Swanson Formation of western Marie Byrd Land linking these two areas east of the Bowers graben³¹. Because of its medial position between disparate geological terrains the Bowers graben is of fundamental importance in the early and middle Palaeozoic evolution of northern Victoria Land, and is a key element in any reconstruction of Antarctica and Australia.

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Factors determining desert dune type

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While most observers recognize four elemental types of desert dunes (longitudinal, transverse, barchan and star¹⁻³) there is little agreement about which factors determine these types. The angular relationships between the resultant of sand shifting winds and both the crest and principal slipfaces of the elemental types have been discussed qualitatively for many decades. These relationships have been quantified but the wide range of dune types that combine the elemental forms, and the juxtaposition of different dune types, show that wind regime is not the only determinant; for example, sand availability has been considered important. We have now quantified the importance of both wind regime and sand availability as determinants of dune type. We show here that the four elemental types occur in areas uniquely defined by both the equivalent sand thickness of sediments held in the dunes and a measure of directional variability of sand-moving winds. Wind strength does not appear to be important, vegetation has an ambiguous role, and the particle size of dune sediments is unimportant.

Two hypotheses recently propounded to explain dune spacing^{4,5} and the geometric similarity of bedforms in rivers, desert dunes and clouds⁶, both appeal to secondary transverse and longitudinal vortices⁷ as the formative mechanism. While the hypothesis of granulometric control of dune spacing^{4,5} did not explicitly attempt to explain the distribution of dune types, the second hypothesis (the geometric similarity argument) drew on the granulometric argument as the prime example. The geometric similarity argument has been fundamentally criticized as mechanically unsound⁸, and may simply reflect the limited array of patterns adopted by natural phenomena⁹. The geometric similarity argument is, therefore, unsound and furthermore the granulometric control hypothesis recently has been shown not to apply to the Australian dune fields and so is not universally applicable¹⁰. More recent data from India and elsewhere reinforce this view (Fig. 1).

If grain size (expressed as the coarse twentieth percentile of crestal sand, P_{20}) is a determinant of dune type, then values of P_{20} and s (dune spacing measured normal to principal slipfaces) could be used to discriminate between dune types. There are 190 data points on Fig. 1 from various dune types in five dune fields, supplemented by 383 measurements of s and 52 measurements of P_{20} (ref. 10). It is quite clear from this figure that dune type is not defined by P_{20} and s .

Sand supply is clearly an important variable³ but the only quantifiable measure of it is equivalent sand thickness (EST), defined as the thickness of a continuous sheet of sand which results from the hypothetical spreading out of dunes, over a specified area. Sand supply and EST are often identical but the possibility of sand moving through dunes into areas outside dune fields¹¹ means that EST and sand supply may not be exactly the same for highly mobile dune fields in areas of high wind energy.

Values of EST for the three main Australian dune fields (Simpson–Strzelecki, Great Sandy, Great Victoria) have been calculated as follows. Blocks of dunes in Australia have statistically homogeneous values of s and dune height (h)¹⁰ over areas of the order of 10 km². Any analysis of relationships between h and s is most conveniently done through $\bar{h}$ and $\bar{s}$ (the mean

values for blocks of dunes). EST will vary with $\bar{h}$ because $\bar{h}$ varies linearly with s (ref. 10). To use $\bar{h}$ to calculate EST we must first calibrate h . The cross-sectional area (A) and h of longitudinal dunes in the three Australian dune fields have been measured from our own surveys in the Strzelecki–Simpson¹⁰ and from levelled seismic traverse lines in all three dune fields. Dunes with at least five surveyed points were used and many more points were usually available.

While dh/dA was initially linear within each dune field, but different between the three dune fields, in the Simpson–Strzelecki h remained essentially constant for values of $h \geq 23$ m, while in the Great Sandy the limiting value of h was ~ 17 m. The sample available from the Great Victoria dune field did not show the same tendency for h to remain constant perhaps because it did not include any large dunes. The range of A from 5,000 to 12,000 m², in the Great Sandy and Simpson–Strzelecki dune fields for $h > 17$ m and $h > 23$ m respectively, implies that dunes higher than these critical values vary widely in cross-sectional shape.

To estimate EST for the blocks of dunes used to calculate $\bar{h}$ and $\bar{s}$, it has not been possible to measure A directly over vast tracts of desert. Instead the linear regression equations relating h to A for the three dune fields have been used to calculate EST for values of h below the critical values. The calculations were made by first computing the average cross-sectional areas (A) for several values of h using the regression equations. Then, for each value of h the average number of dunes occurring along a 10-km traverse across the dunes was calculated using the regression equation relating $\bar{h}$ to $\bar{s}$ for each dune field. Finally, each of these values was multiplied by the appropriate average value of cross-sectional area, previously calculated from the relationship between $\bar{h}$ and A , to obtain a value of EST for each value of h . Since the number of dunes starting (upwind ends) and finishing (downwind ends) in an area of 100 km² is approximately the same, the calculated values of EST apply to areas as well as traverses. The results of this analysis are given in Fig. 2.

By deliberately excluding some accurately levelled dunes from the data used to construct the regression equations relating h to A , it has been possible to check the values of EST calculated using the average data by direct measurement of A for the excluded dunes. The calculated values of EST shown in Fig. 2 underestimate the measured values by 15% on average. The discrepancy arises because calculated values of EST are based on the regression equations of $\bar{h}$ against A for values of h less than the critical values. This calculation ignores the high values of A that can occur with similar values of h above these critical heights. This problem is unavoidable because A cannot be accurately measured for a large number of dunes, as noted earlier.

Additional values of EST and h have been derived by placing a grid over maps of EST and h (ref. 5) for the Erg Oriental, the Erg Occidental and the Issaouane-N-Irrarren (all in Algeria), and over maps of dune types¹² for the same areas. EST in barchans has been accurately measured in Peru² and, using these authors' method of volume calculation, we have estimated values of EST for barchans in the Thar. For the transverse dunes of the Nebraska Sandhills, EST has been calculated previously¹³ and h can be taken from ref. 14. Mean values of both variables have been calculated because their exact covariation is not clear from the data available. Values of $\bar{h}$ and EST have been extracted for the parabolic clusters (which are here considered to be transverse dunes) of the Thar by analysis of published maps and some field checking. These results have been calculated by making the simplification that the dunes are triangular in profile parallel to the direction of dune movement, and the shape of the bowl within each parabolic element has been determined from a limited number of profiles measured across the dunes. Parabolics in the Victorian Mallee (Australia)¹⁵ have been surveyed and values of EST and $\bar{h}$ estimated. Regression equations relating s , h and width of linear dunes in the Namib¹⁶ have been used to derive the

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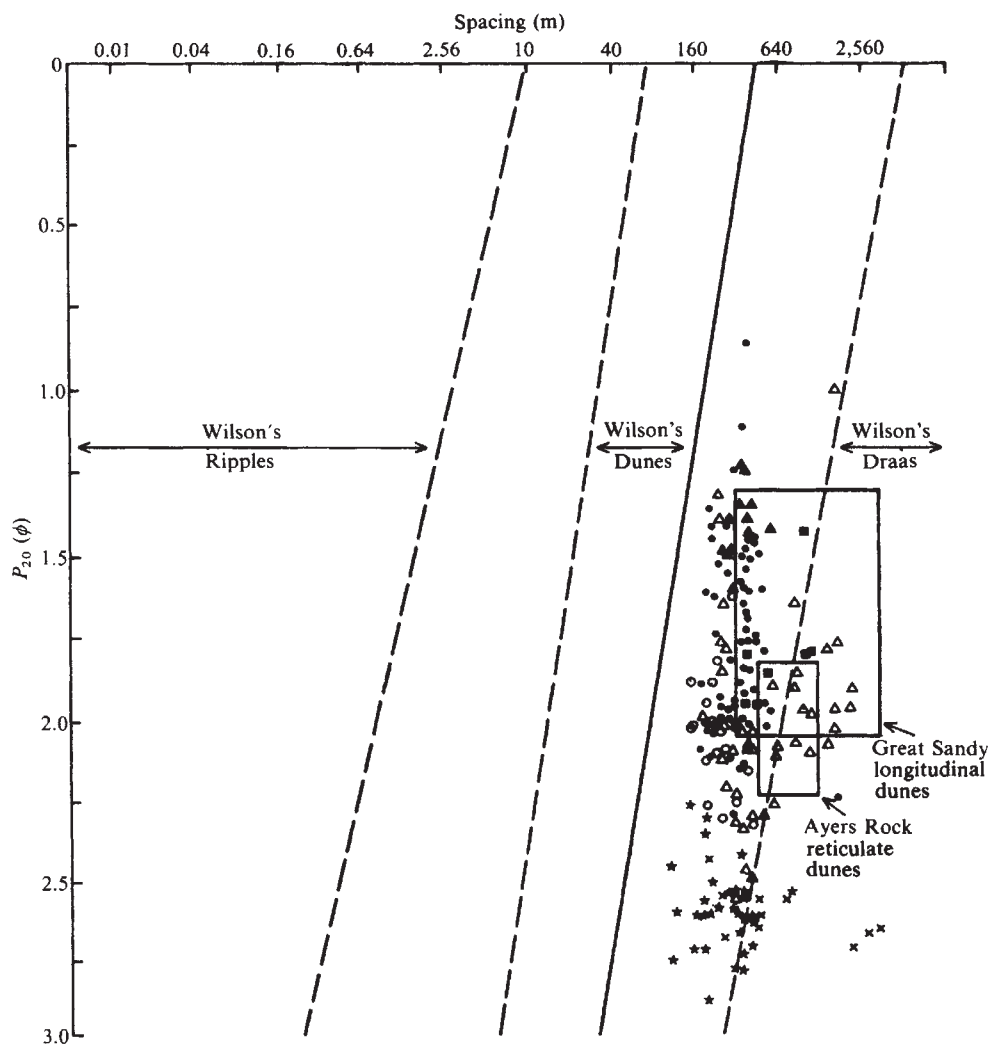


Fig. 1 Crestal sand coarse twentieth percentile (P_{20}) and dune spacing (s) diagram showing Wilson's (ref. 5) original categories and new data from various parts of the world. Australian locations: ●, Strzelecki (longitudinal); △, Simpson (longitudinal); ■, Mt Rescue (parabolic/transverse); ○, Kulwin-Innesowen (longitudinal); ▲, Manya; Great Victoria (longitudinal) and Ayers Rock (reticulate). ★, Peru (barchans); ×, Thar (parabolics).

approximate average relationship between h and EST by assuming equal spacing and constant shape for the dunes.

The h and EST data from the world's dune fields are presented in Fig. 3, and it is clear that h -EST relationships vary between dune fields. In all cases, however, EST increases with $\bar{h}$ or h . In Australia the differences between the h -EST curves for the different dune fields (Fig. 2) result from differences in sand supply and substrate type (noted in ref. 3), and these two factors are likely to be important in the other dune fields represented in Fig. 3.

From the data in Figs 2 and 3 it is not possible to identify the role of EST in determining dune type. Therefore, all values of EST for each elemental dune type have been averaged (see Fig. 4). The values of EST for transverse and star dunes are statistically identical, as are the values for linear and barchan dunes at $P = 0.001$ using a Mann-Whitney U test. Furthermore, values of EST for star and transverse dunes are statistically different from values for linear and barchan dunes, also at $P = 0.001$. EST is obviously a significant variable but it is not the only determinant of dune type.

Dune-forming wind regimes have been quantified in the world's dune fields in a systematic analysis¹⁷ using calculations based on a modification of Bagnold's sand transport equation². The magnitude of the vector resultant of drift potential (DP) is known as the resultant drift potential (RDP), and a measure of the directional variability of dune-forming winds is given by RDP/DP. Low values of this ratio reflect high variability¹⁷.

Where dunes are unequivocally mobile the present dune-forming winds should be reflected by RDP/DP. Values of this ratio calculated for stations in or near relict or partially stabilized dune fields may not necessarily represent the dune fields' formative wind regimes. Many dune fields were actively forming

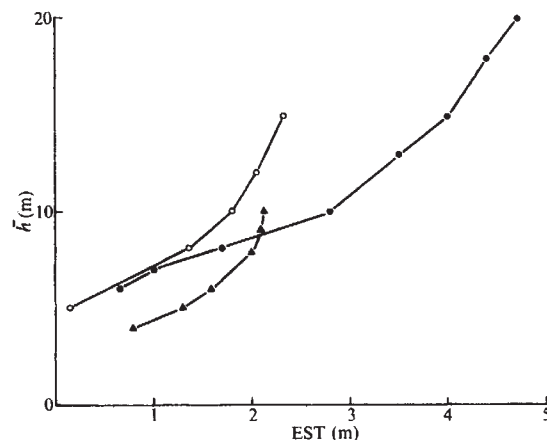


Fig. 2 Calculated equivalent sand thickness (EST) and mean dune height (h) for Australian dune fields. ○, Great Sandy; ▲, Great Victoria; ●, Simpson-Strzelecki.

at the last glacial maximum some 18 kyr BP (ref. 18) in atmospheric conditions that differ from those of today^{19,20}. Despite this, dune trends in Australia deviate only slightly from weighted resultant sand-moving winds in the Simpson Desert²¹. Major deviations between RDP and dune orientation are not very common in other dune fields^{17,22}. In our analysis we have omitted data from areas of major deviation and so modern values of RDP/DP are reasonable estimates of dune-forming winds. Because of the paucity of meteorological data for most dune fields we have not been able to calculate RDP/DP and

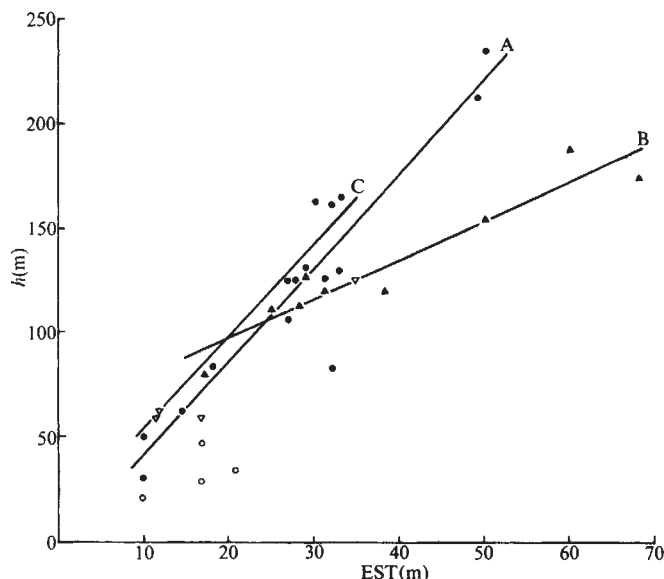


Fig. 3 Dune height and equivalent sand thickness (EST) for various dune fields. ●, Erg Oriental (A); ▲, Issaoune-N-Irarraren (B); ▽, Erg Occidental; ○, other dune fields (Australia, Thar, Peru, Nebraska) mean values; Namib (C).

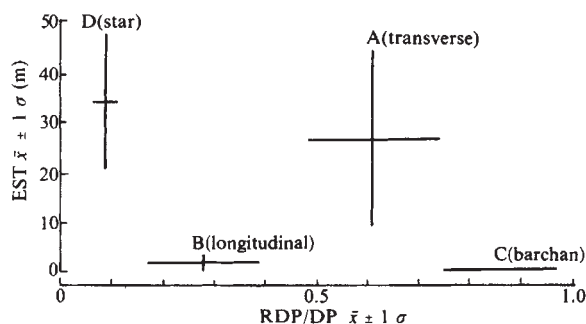


Fig. 4 Statistically significant ($P=0.001$) separation of the four elemental dune types by means of the two variables: equivalent sand thickness (EST) and a measure of wind directional variability (RDP/DP). The $\bar{x} \pm 1\sigma$ for EST in barchans is 0.02 ± 0.005 m ($n=8$).

EST for the same dune fields in all cases. In Fig. 4 the RDP/DP values are from ref. 4.

The combination of RDP/DP and EST in Fig. 4 produces clear discrimination of the elemental dune types. Using the Mann-Whitney U test²³, the data show that star dunes occur in wind regimes more variable than those which produce linear dunes, at $P=0.001$, and transverse dunes occur in more variable regimes than barchans at $P=0.001$.

Other variables considered to be important are wind strength^{14,17,24} and vegetation²⁴. Using DP (from ref. 17) as a measure of wind strength we find that the elemental dune types occur randomly throughout the range of values between 33 and 658 vector units.

Vegetation is obviously significant in the parabolic dune form^{24,25} but it is not clear whether, apart from that, it significantly affects the development of one dune type in preference to another or rather is itself partly controlled by the mobility and form of the dune. Given the significance of Fig. 4, vegetation must be considered a modifying factor rather than a determinant.

We conclude that barchans occur where there is very little sand and almost unidirectional winds; transverse dunes occur where sand is abundant and the wind is moderately variable; longitudinal dunes occur where the winds are more variable but there is little sand; and star dunes occur where sand is abundant and wind variability is at a maximum. These results are intuitively sensible and conform to most of the qualitatively

expressed opinions of other authors (see refs 1, 3 and 26). These conditions for the development of different dune types are now quantified using the best available data. One important implication of these results is that RDP/DP reflects the significance of synoptic-scale flow, in the development of dune type, rather than secondary flow within the boundary layer appealed to in various hypotheses^{4,5,7}.

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Satellite and ship studies of coccolithophore production along a continental shelf edge

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Each year since the Coastal Zone Color Scanner (CZCS)¹ was launched on the Nimbus 7 satellite in November 1978, extensive patches of water giving strong reflectance of visible light have been observed during the early summer along the outer margin of the north-west European continental shelf between 45 and 60° N (refs 2, 3). Various hypotheses including coccolithophores, phytoplankton with external calcified plates or coccoliths, were suggested to explain a comparable feature on Landsat images for July 1977⁴. To test these, we report here observations made from French and UK research vessels in 1982, using unprocessed CZCS images supplied by the University of Dundee and Centre de Meteorologie Spatiale in Lannion to locate suitable sampling areas immediately before and during the cruise, and atmospherically corrected data from the European Space Agency for subsequent analysis and calibration of the reflectance signals. The high reflectance was found to be

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caused by a surface bloom of the widespread coccolithophore, *Emiliania huxleyi* (Lohm.) Hay et Mohler, which also produced abundant detached coccoliths. This finding is of relevance to recent work both on the ecology of coccolithophores⁵, including their importance in the sedimentation of biogenic carbon^{6,7} and lipids⁸ and in palaeoclimatic studies⁹, and on the development of reliable remote sensing techniques for the estimation of chlorophyll in oceanic waters¹⁰.

On the Celtic and Armorican Shelf regions, the 'high reflectance' water appears first during the latter half of April or early May¹¹ and persists until mid or late June either as a series of discrete patches (Fig. 1a, c) or as extended bands (Fig. 1b) close to and inshore of the 200-m contour. The zone of cool water that marks the shelf break front³ generally defines their outer limit (see Fig. 1d) at this time of year. The larger patches are identifiable for at least 3 weeks; the one investigated in May 1982 (Fig. 1c, d) was first detected at sea on 9 May and by satellite on 15 May and was last seen on the CZCS image for 8 June just before a period of cloudy weather. Later in the summer smaller patches occasionally develop further onto the shelf (see, for examples, Fig. 1c, and ref. 2, Fig. 1a).

The plankton and hydrographic observations made on the outer shelf in May 1982 are summarized in Fig. 1d and Table 1. By the end of the month surface temperatures were generally 12.5–13.5 °C, with the lowest values close to the 200-m contour, and surface nitrate levels were <1 µM on the shelf where a shallow thermocline had developed but still relatively high close to the shelf break and in the oceanic waters to the south-west. The reflective water, in which the phytoplankton was dominated by the coccolithophore *Emiliania huxleyi* (Fig. 2a) at densities up to 8,500 cells ml⁻¹, was visually turbid, with a pale turquoise colour apparent even in aerial photographs taken from altitudes up to 10 km. Each cell had an average of 20 attached coccoliths so that the detached liths (up to 10⁶ ml⁻¹, Fig. 1d) represented an estimated 37–64% of the total number in the water. Chlorophyll *a* levels were generally between 1.0 and 1.5 mg m⁻³ within the coccolithophore bloom compared with lower values in the nutrient depleted shelf waters and higher values (up to 2.5 mg m⁻³) along the shelf break to the south-east where

diatoms and dinoflagellates were dominant. It was not possible, therefore, to define the limits of the coccolithophore population by *in vivo* measurements of chlorophyll fluorescence.

Surface water samples collected at stations 5 and 7 for analyses of particulate material gave calcium concentrations of 450 and 31 mg m⁻³ respectively, equivalent to 1.8 and 2.0 pg Ca per lith. The latter values must be considered maximal since coccoliths in faecal pellets and other derived material (see Fig. 2b,c) would not have been included in the microscope counts, and they are higher than the 0.55 pg Ca lith⁻¹ reported for cultured cells¹². Note that the corresponding carbonate-carbon concentrations of 135 and 9 mg C m⁻³ form a relatively low proportion of the total particulate carbon (Table 1).

The reflectance values detected by the CZCS for the coccolithophore-rich water are much greater than those measured *in situ* for other shelf waters containing comparable amounts of chlorophyll (Fig. 3a). A significant correlation was found between reflectance measured by each of the CZCS channels 1–3 (centred at 443, 520 and 550 nm respectively) and the surface abundance of coccolithophores (Fig. 3b), and under the microscope the particulate material was seen to consist almost entirely of cells and coccoliths of *E. huxleyi*. Thus the high reflectance appears to have been due to a combination of backward scattering by the coccoliths and of relatively low absorption by plant pigments and other constituents of the water¹³. The difference is most marked at the shorter wavelengths (channels 1 and 2—see Fig. 3a) probably due in part to increased absorption by yellow substances and suspended matter in the more coastal waters^{14,15}. The shelf break coccolithophore populations are therefore recognizable on the satellite images by the intensity and spectral composition of the reflected light.

The relative contributions of the living cells of *E. huxleyi* and the detached coccoliths to the reflectance signal are not known. Cultures have been shown to give low values of backward scattering¹⁶, but in the laboratory *E. huxleyi* tends to produce relatively few coccoliths at salinities >30‰ (ref. 17). Further studies of the optical properties of cell and lith suspensions are required for proper interpretation of the satellite

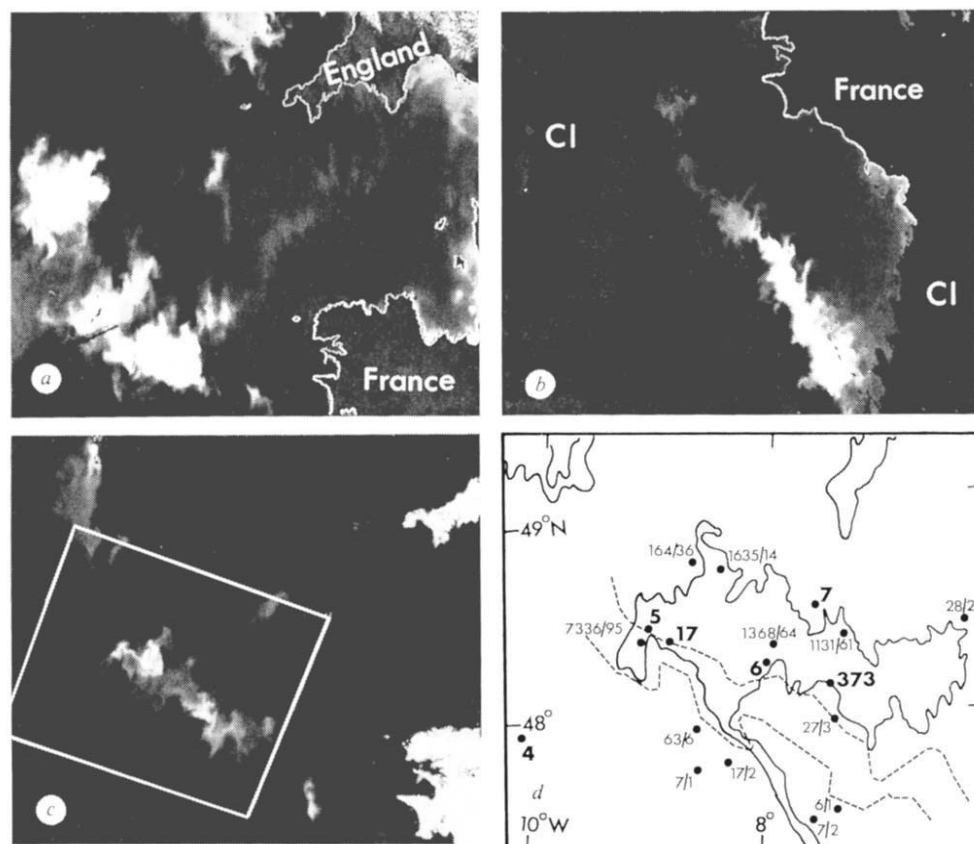
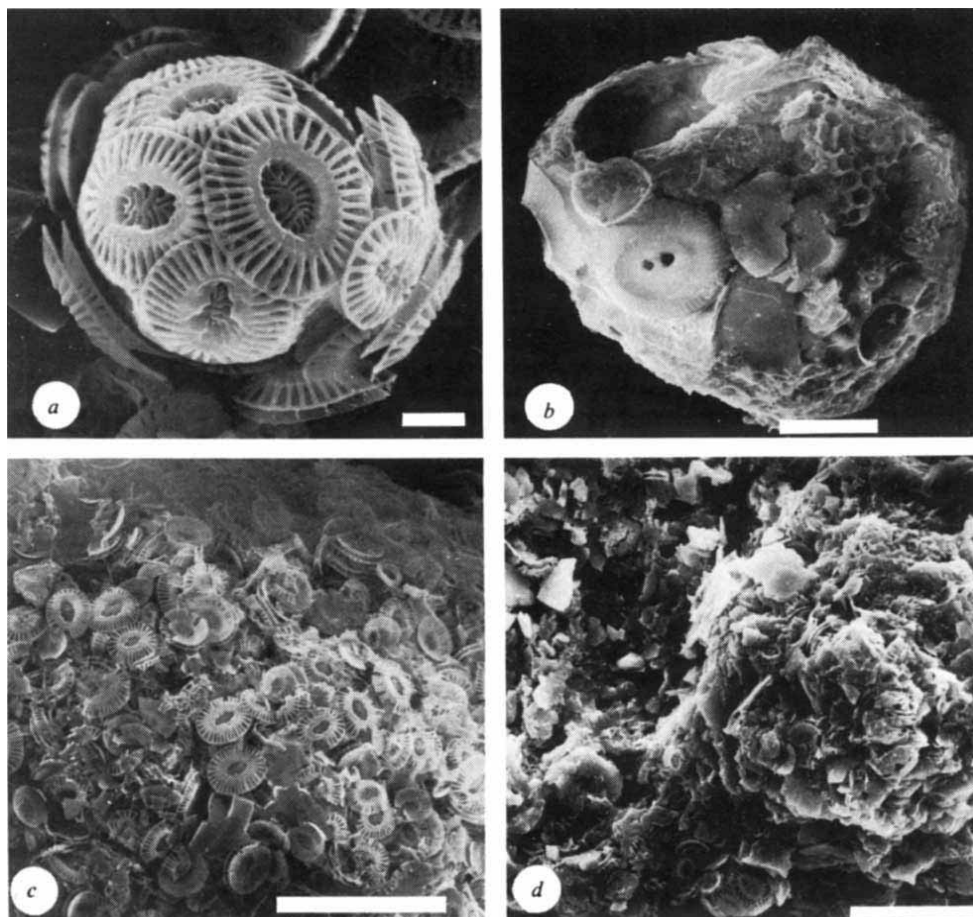


Fig. 1 a–c, CZCS Channel 3 (550 nm), atmospherically corrected³², images of the Celtic and Armorican Shelf regions for 19 June 1979 (orbit 3293); 10 May 1981 (orbit 12843); and 29 May 1982 (orbit 18150). CI, clouds. Time degradation of the CZCS sensors was taken into account (see ref. 2). The coccolithophore water gave significant reflectance at 670 nm so that values from adjacent pixels were used for the aerosol correction. A single value (1.1) for the Angstrom exponent in the aerosol term could be applied in this instance, as the water-leaving radiance was much greater than the atmospheric aerosol component. d, Sampling locations (●) for 9 May (station 373) and 26–28 May 1982 (other stations) in relation to the position of the surface coccolithophore bloom on 29 May 1982 (see Fig. 1c). The stations listed in Table 1 are shown as bold numbers. Values for the other stations represent *E. huxleyi* cell and detached lith ($\times 10^{-3}$) numbers per ml respectively. The position of the band of cool water at the shelf edge, derived from the channel 6 IR image for 29 May, is indicated by the dashed lines.

Fig. 2 Scanning electron micrographs of *a*, *E. huxleyi* from station 5, showing outer layers of partly detached coccoliths; *b* and *c*, tintinnid lorica incorporating coccoliths of *Coccolithus pelagicus* (Wallich) J. Schiller and *E. huxleyi*, and diatom fragments; and part of a copepod faecal pellet consisting almost entirely of coccoliths of *E. huxleyi* (both were collected from coccolithophore water on 28 May 1982); and *d*, coccoliths in surface sample of sediment from 600 m, collected 48°30' N 09°52' W on 17 March 1983. Scale bars 1 μm (*a*) and 10 μm (*b,c,d*).



data, especially since detached liths sink very slowly from the surface layers⁶ and may be the main cause of reflectance during the later stages of any bloom.

The presence of coccoliths affects CZCS algorithms for the estimation of chlorophyll in surface waters both by causing significant reflectance at 670 nm (channel 4), which has to be taken into account in the atmospheric corrections^{10,18}, and by influencing the coefficients used in standard channel ratio or difference equations for chlorophyll. Although the first problem can be resolved by correcting the channel 4 signal by correlation with those for channels 1–3, the second presents many difficulties as coccolithophores are an important but variable component of the phytoplankton in all the oceans between the Arctic and Antarctic convergences^{19,20}. For example, it now seems likely that the bright features in the North Atlantic on a Landsat MSS band 4 image²¹ and at the shelf edge on a CZCS channel 1 image of the Middle Atlantic Bight (Fig. 9 in ref.

10) show surface coccolithophore populations in which reflectance may or may not have covaried with surface chlorophyll levels.

The coccolithophore bloom in May 1982, although associated with relatively low levels of chlorophyll and therefore, by implication, of organic carbon production, was certainly potentially important as a source of inorganic carbonate for sedimentation particularly along the upper slope region where bottom mixing due to tides decreases sharply²². The surface cell densities were lower than those reported for some coastal blooms of *E. huxleyi*^{23,24}, but integrated values for the water column at station 5 (Table 1) give $>3 \times 10^{11}$ *E. huxleyi* cells m^{-2} and 40 g $\text{CaCO}_3 \text{m}^{-2}$ for the upper 60 m alone which are as high as any previous comparable estimates. A conservative average value of 10 g $\text{CaCO}_3 \text{m}^{-2}$ for the single patch (7,200 km^2 , Fig. 1c, d) is equivalent to 7.2×10^4 tonnes of calcite without taking into account either material below 60 m (note the abundance of *E.*

Table 1 Coccolithophore and hydrographic data for May 1982

Station	Water depth (m)	Sample* depth (m)	<i>E. huxleyi</i> (cells ml^{-1})	Detached coccoliths (ml^{-1})	Chl <i>a</i> (mg m^{-3})	Particulate carbon (mg m^{-3})	Nitrate (μM)	K† (m^{-1})
373	152	0	4,200	—	0.54	—	5.6	—
		145	1,000	—	0.33	—	6.2	—
4	4,000	0–20	20	1,800	0.81	171	4.2	0.11
5	175	0–16	8,516	78,300	1.38	291	2.3	0.24(0.57)
		0–60	5,258	60,000	1.08	369		
6	184	0–20	2,183	50,498	1.67	283	1.7	0.31(0.68)
		0–60	1,227	24,000	1.37	213		
7	170	0–20	324	7,000	0.90	265	0.3	0.15
17	190	0–20	959	29,300	1.35	191	0.7	0.20(0.26)
		0–60	1,002	35,700	0.79	167		

* The water was sampled with bottles at Station 373 on 9 May, and with a pump profiling system at the other stations on 26–28 May.

† K, Extinction coefficient, was measured with a 350–700 nm band photometer. Values in parentheses were derived from the relationship 1.7/secchi disk depth (m).

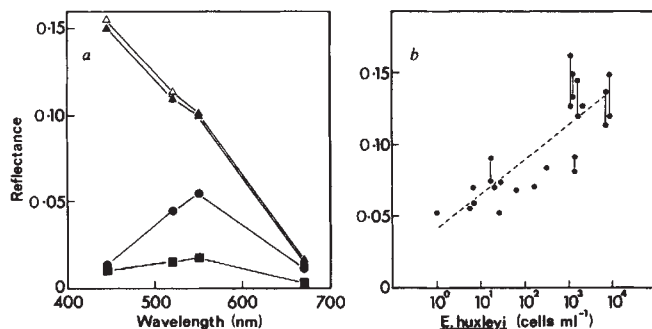


Fig. 3 *a*, Reflectance spectra comparing coccolithophore water on 19 June 1979 (Δ) and 29 May 1982 ($\blacktriangle$) (see Fig. 1*a, c*) with waters carrying low and high loads of suspended sediment in the western ($\blacksquare$) (ref. 2) and eastern ($\bullet$) (M.V., unpublished data) English Channel respectively. In each case, except 19 June 1979 for which there are no field data, the surface chlorophyll *a* levels were 1.5–2.0 mg m⁻³. The coccolithophore reflectances were derived from the corrected CZCS scenes (see notes to Fig. 1), and the English Channel reflectances from field measurements of upwelling irradiance. *b*, Plots of reflectance (CZCS channel 1, 443 nm) against surface cell counts of *E. huxleyi*. The regression line $R_{443} = 0.0411 + 0.0246 \log(\text{cell})$ ($r^2 = 0.67$) yields the algorithm $(\text{cell}) = 2.1 \times 10^{11} (R_{443})^{8.7}$ where (cell) is the concentration ml⁻¹ of *E. huxleyi*. To take into account uncertainties due to difficulties in locating the sampling positions on the satellite image, and to time differences between sampling and the satellite pass, the minimum and maximum reflectance values for 5×5 pixel squares around each sampling position are shown as vertical bars. The absence of a bar indicates uniform reflectance over this area.

huxleyi down to the bottom at Station 373, Table 1) or any net sedimentation and production before and after the period of observations. As the blooms extend along much of the shelf margin during the early summer each year (Fig. 1*c*; and CZCS image for 16 June 1982 of the area to the west of Scotland), the total annual calcite production by coccolithophores for the north-west European shelf edge is likely to be at least an order of magnitude greater than this.

The effects of tides and wind²² on the dispersion of the particulate CaCO₃ are uncertain. Sequences of satellite images for the Celtic Sea each year showing surface distribution patterns persisting for several weeks indicate that, for this region at least, horizontal processes may be relatively unimportant although surface plumes of high reflectance water (Fig. 1*c*) occasionally extend into the Bay of Biscay and subsurface nepheloid layers containing coccoliths²⁵ have been observed in slope waters. The incorporation of coccoliths into the skeletons of larger organisms, copepod faecal pellets²⁶, and into fine sediments below 200 m (Fig. 2*b, c, d*) suggest that there is net sedimentation of calcite occurring in conditions of negligible re-dissolution (the lysocline for calcite in the North Atlantic⁷ is ~4,600 m) and at rates much in excess of the mean yearly global value of 0.41 g m⁻² inorganic carbon (this represents about three-quarters of the total carbon presently being buried in oceanic sediments)²⁷.

The continental slopes are now recognized as major areas of net carbon deposition²⁸. Thus, due both to their location and to the favourable conditions for preservation of the calcite, the shelf-edge coccolithophore populations are likely to have a particularly important role in the transfer of carbon from the atmosphere to the oceanic sediment. The fact that these blooms have not been recognized as regular events in an area that has been relatively well studied by oceanographers for the past 100 yr (there are earlier reports of abundant coccolithophores close to the shelf break^{29,30} and on the Rockall Bank³¹) emphasizes the important contribution that satellite colour scanners can make to investigations of large scale biological processes in the oceans, especially if the images can be available for the planning of research cruises.

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Genesis of humus B horizons in hydromorphic humus podzols

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A distinctive podzol B horizon, characterized by accumulated humus, low-iron content, lateral uniformity in appearance and composition and frequently hard cementation, develops at the top of the water table in quartz sands on coastal plains in the subtropics and tropics. Soils with such B horizons are separated from freely-drained podzols in many classifications, such as groundwater podzols¹, humus podzols², hydromorphic humic podzols³, aquods and humods⁴, because of a distinctive morphology and possible differences in genesis. However, while an association between the humus-rich B horizon (Bh) and the underlying water table has long been recognized, most explanations offered for the mode of formation of the Bh have been essentially similar to those for the Bh (iron-rich Bh) horizons in freely-drained podzols. These do not provide a satisfactory

explanation of the mechanism whereby organic matter is precipitated in an environment that is virtually free of sesquioxides. We suggest here that such Bh horizons may form as the result of coprecipitation in the B horizon between organic-rich surface waters and aluminium from the groundwater. Further, such a mechanism can lead to the development of organic-cemented hardpans, commonly called coffee rock^{3,5}, that are so frequently associated with hydromorphic podzols in both temperate and tropical regions³.

The Bh horizons of hydromorphic humus podzols differ from the Bh horizons of freely-drained podzols in several features that are particularly obvious in the soils of the dune systems and sandplains of coastal Queensland⁶. Comparing profiles having similar depths of A horizon (0.5–1.5 m), the Bh horizon of humus podzols differs from the Bh horizon of humus-iron podzols in four respects.

(1) Site of formation—the Bh forms in clean quartz sands at about the level of the permanent water table, whereas the Bh horizon forms in the upper part of freely-drained sands that are coated with sesquioxides.

(2) Morphology—the upper boundary of the Bh is even and lacks tongues of A₂ that commonly penetrate into Bh horizons; also, the organic precipitates in the Bh appear to be evenly distributed laterally whereas those in Bh horizon occur as streaks and patches.

(3) Composition—the organic precipitates of the Bh are almost entirely Al-humates and composition laterally is relatively constant, whereas the Bh horizon consists of Fe–Al humates with a high lateral variability in composition.

(4) Consistence and porosity—Bh horizons may become hard and cemented (coffee rock) with very low porosity so that water perches above them during the wet season, and issues from the soils as so-called 'black waters', coloured with humus derived from decomposing litter⁷. In contrast, the Bh horizons of freely-drained podzols are friable to firm⁶ and permeable; also, the cemented Bh horizons are much more resistant to erosion and similar to sandstone in exposures along the beach where they have also been termed sandrock^{8,9}.

Despite these differences, the mode of formation of the Bh horizon in hydromorphic podzols has mostly been considered as essentially similar to that of well-drained podzols^{3,10}. The traditional view is that aluminium and iron released by weathering in the A horizons are translocated as organic complexes and deposited in the B horizon where there is an increase in sesquioxides, although in situations where reducing conditions prevail the iron is lost from the profile in iron(II) form. However, most hydromorphic humus podzols have formed in sands with very low initial contents of sesquioxides and/or lack weatherable minerals that might provide continuing inputs of iron and aluminium. Therefore, while free sesquioxides are essential to the formation of Bh horizons in freely-drained podzols, a different process is probably involved in the development of Bh horizons in hydromorphic humus podzols. Moreover, major differences in morphology and micromorphology between Bh and Bh horizon suggest different mechanisms of precipitation.

In hydromorphic humus podzols (Fig. 1a), organic precipitates fill the space between clean quartz sand grains in a manner that suggests precipitation from solution. In freely drained podzols (Fig. 1b) the sand grains are coated with darker organic matter and paler (yellowish) sesquioxide coatings, and the space between the grains is largely unfilled. Although the Bh horizons of humus-iron podzols may also contain organic matter not associated with grain surfaces, this is usually in discrete forms such as root residues, faecal pellets and shed coatings^{10,11}, none of which features in the Bh horizons of hydromorphic humus podzols.

In reassessing the processes that form podzols, it has been proposed that, in humus-iron podzols on coarse parent materials, organic matter migrates through the A₂ horizon in soluble and colloidal form and is adsorbed or precipitated at the top of the B horizon on surfaces that initially carried coatings of sesquioxide, imogolite or allophane, such coatings still being

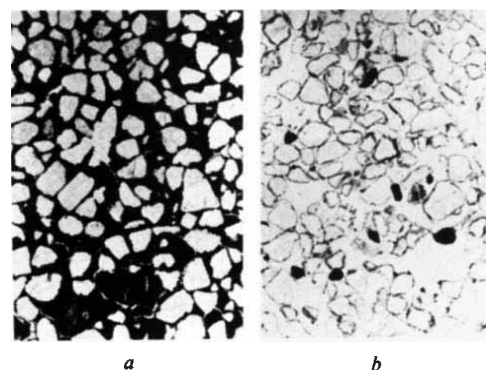


Fig. 1 Photomicrograph of: a, the Bh horizon from a hydromorphic humus podzol, Cooloola, Queensland, showing precipitated organic matter filling the spaces between quartz sand grains; b, the Bh horizon of a freely-drained podzol, Cooloola, Queensland, showing quartz grains coated with darker organic matter and paler sesquioxide; spaces between grains are largely unfilled.

present in the underlying (Bs) horizon¹²; or as it moves down in the leaching water it takes up more and more iron and aluminium from the lower sesquioxide-rich horizons until its original charge is neutralized, leading to the mutual precipitation of the metals and organic matter¹³.

Both processes imply the availability of both iron and aluminium compounds (sesquioxides) in the B and lower horizons. However, the parent materials of many hydromorphic humus podzols initially contain little sesquioxide and there is no evidence of sesquioxide coatings (enrichment), nor could they form in the zone of permanent saturation. Pyrophosphate-extractable aluminium varies in Bh horizons up to 0.5% whereas the pyrophosphate-extractable iron is <0.005%; the organic matter is readily extracted in alkaline conditions and more than 90% of the extractable fraction is in the form of humic acid, indicating that it has been precipitated from colloidal solution, and not from true solution.

Laboratory experiments^{13,14} have shown that the addition of aluminium in solution to solutions of dispersed organic matter leads to mutual precipitation when the overall carbon-to-aluminium ratio falls below 20 within the range pH 4–4.5 (ref. 14). It has already been noted⁷ that conditions which prevail on mixing of organic-rich 'black' waters with aluminium-charged 'white' waters lead to increased stability of the organic colloid rather than coprecipitation. A simple mixing of 'black' and 'white' waters therefore cannot result in the formation of a stable precipitate. The 'white' water can, however, act as a source of ionic aluminium for the gradual increase in aluminium in these podzols.

Formation of the Bh horizon can be initiated when part of the B horizon of a humus-iron podzol reaches the water table or where organic matter is deposited in the oscillating zone of the permanent table during seasonally dry periods. Fluctuations in the water table will result in periods of inundation of this organic matter and begin the cycle. Thereafter, penetration of ionic aluminium from the water table into the semipermeable¹⁵ B horizon by diffusion will result in a slow increase in the aluminium content, thus reducing the C/Al ratio. While this ratio remains high, the aluminium becomes bound to the organic matter and the diffusion is virtually one-way. Organic matter can still penetrate provided the B horizon remains diffuse and is precipitated by the increased aluminium concentration. Simple laboratory experiments have determined that this mechanism for the fixation of aluminium and carbon is feasible. This process results in the pores between sand grains becoming filled with organic matter of a high aluminium content. A further continuation of the process results in a net transfer of aluminium from the 'white' to the 'black' water and a gradual building up of the Bh horizon. The rate and extent to which these horizons can grow will depend on the thickness and degree of cementation of the pan.

Duchauffour³ recognizes a class of 'hydromorphic humic podzols' characterized by the presence of thick Bh horizons, dominated by aluminium humate precipitates, at around the level of the water table in quartose sands, and gives references to many examples from both temperate and tropical climates. The mechanism of formation proposed here, that is, precipitation of organic-rich surface waters by Al-containing groundwater, is considered a more satisfactory explanation of the location, depth and amounts of precipitates formed than that proposed by Duchaufour, which postulates migration of Al organic complexes from the overlying A₂ horizon, but gives no satisfactory mechanism for their precipitation, nor for the great excess of humic acids over fulvic acids in such horizons, nor, indeed, for the complete infilling of intergranular pores in the sands in the most extreme cases.

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Loosening of paranodal myelin by repetitive propagation of action potentials

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We have previously shown by X-ray diffraction techniques that the stability of the myelin lattice is decreased by repetitive stimulation of the nerve¹. We now show that in rat myelinated fibres this structural change is accompanied by the appearance of voltage-sensitive potassium channels which do not normally participate in the generation of the action potential. Since it has previously been shown that structures associated with potassium channels are found in the internodal and paranodal axolemma membrane but are normally concealed under the myelin sheath^{2,3}, we suggest that the acceleration of myelin swelling after repetitive propagation of the action potential may be due to the opening of paranodal axoglial junctions. To our knowledge this is the first demonstration that nerve activity may regulate the structural properties of the paranode.

The two sciatic nerves of an adult rat were carefully dissected and mounted in a thermostatically controlled chamber at 37 °C for stimulation and monophasic recording of the compound action potential (CAP). For equilibration, the nerves were incubated for 20 min in normal Ringer solution (NR) containing: 137 mM NaCl, 5 mM KCl, 1.1 mM MgCl₂, 1.25 mM CaCl₂, 1.1 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 12.5 mM NaHCO₃ and 10 mM glucose. This solution was bubbled continuously with a gas mixture of 95% O₂-5% CO₂. After the initial equilibration, one of the nerves was stimulated supramaximally at 200 Hz

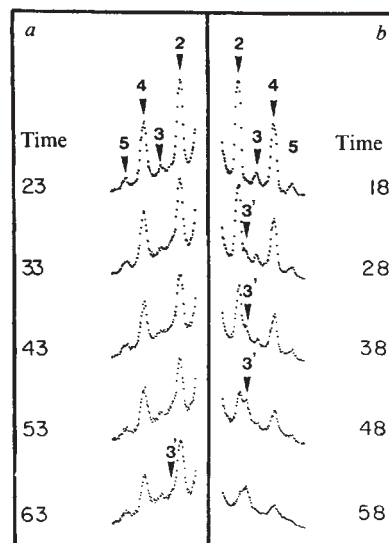


Fig. 1 Kinetics of swelling in distilled water of: *a*, myelin from a sciatic nerve incubated for 1 h in NR; *b*, myelin from the other sciatic nerve of the same rat after 1 h of supramaximal stimulation at 200 Hz. Each pattern was accumulated over 5 min. The times indicated in the two sequences represent the elapsed time (min) in distilled water at the onset of each data collection period. The initial spectra in *a* and *b*, recorded at 23 and 18 min, respectively, are very similar to each other, indicating that changes produced in the internodal myelin membrane structure by nerve activity are negligible at low resolution, in agreement with previous observations^{1,7}. Reflections 2-5 from the native structure and the third-order reflection from the swollen lattice (3') are indicated by arrows. Note that the repetitive propagation of action potentials significantly accelerates the rate of subsequent myelin swelling: in *b*, the process is almost complete within 58 min for the stimulated nerve exposed to distilled water, whereas in *a*, the third-order reflection from the swollen lattice (3') has scarcely begun to appear at 63 min for the unstimulated nerve exposed to distilled water.

for 1 h while the other was kept unstimulated in NR for the same length of time. At the end of the stimulation period, both nerves (stimulated and unstimulated) were mounted in a holder provided with two separate perfusion systems, by means of which the nerves were immediately perfused with distilled water. Myelin lattice swelling was followed by dynamic X-ray diffraction. The techniques for recording sequential X-ray diffraction patterns from biological specimens have been described in detail elsewhere^{1,4-7}. Progress of swelling was followed by changes in intensity of the third-order reflection of the swollen lattice related to the intensity of the second-order reflection from the native structure.

Figure 1 shows, as an example, the kinetics of myelin lattice swelling in distilled water for a pair of sciatic nerves dissected from the hindlegs of a rat. In agreement with previous observations carried out in toads¹, the rate of swelling in the stimulated nerve (Fig. 1*b*) was greater than that in the unstimulated nerve (Fig. 1*a*), indicating that, in rats, the repetitive propagation of action potentials decreases the stability of the myelin lattice.

The electrophysiological experiments complementary to the above structural work were also carried out in rat sciatic nerves that had been treated in an identical fashion to those studied by X-ray diffraction. The nerves were dissected and mounted in a thermostatically controlled chamber in such a way that stimulation was produced at the distal end, and the CAP was recorded at the proximal end. Potassium channel activity was detected by determining the CAP sensitivity to 1 mM of 4-aminopyridine in normal Ringer (4-AP). The rationale for using this drug, which blocks voltage-sensitive potassium channels, is that, if potassium currents are contributing to repolarizing currents during the action potential, 4-AP will delay the onset of repolarization in individual fibres and consequently the duration of the CAP will increase⁸⁻¹¹. The sensitivity of the nerves

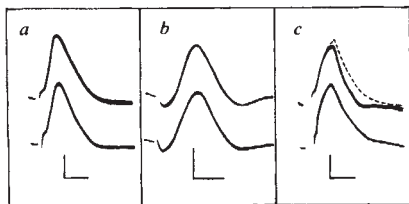


Fig. 2 Compound action potential (CAP) of three rat sciatic nerves, recorded monophasically, before (upper records) and after (lower records) a 15-min exposure to 1 mM 4-aminopyridine (4-AP). Calibration bars are 1 mV (vertical) and 1 ms (horizontal). *a*, Freshly dissected sciatic nerve; 4-AP has no significant effect on CAP, showing that potassium channels are not electrically detected at the Ranvier node. *b*, Unstimulated nerve left in the chamber for 1 h in NR; 4-AP has no significant effect on CAP. This experiment was carried out as a control for *c* (see below) and shows that a 60-min preincubation did not significantly change the sensitivity of the nerve to a 15-min exposure to the drug. *c*, Sciatic nerve stimulated supramaximally for 1 h at 200 Hz; 15 min treatment with the drug produced a significant widening of the CAP, suggesting that nerve activity has the effect of exposing the potassium channels which, after stimulation, are electrically detected at the Ranvier node. For comparison, a CAP showing the effect of 4-AP (lower tracing) has been superimposed (dotted line) on that recorded in NR before drug treatment (upper tracing).

to 4-AP was quantified as the effect on CAP duration at half-amplitude of a 15-min exposure of the nerve to 1 mM 4-AP.

Three groups of experiments were performed. The first (Fig. 2*a*) considers freshly dissected nerves. The sciatic nerve was mounted in the moist chamber and allowed to equilibrate for 20 min in NR, then stimulated for 1 min at low frequency (1 stimulus every 5 s). The corresponding CAP is shown in Fig. 2*a*, upper tracing. The nerve was then perfused with 4-AP for 15 min and stimulated again at the same low frequency. The CAP registered from the 4-AP-treated nerve is shown in Fig. 2*a*, lower tracing. The effect of 4-AP was quantified as indicated above. Table 1 summarizes the results of 12 experiments and indicates that the drug has no significant effect on the CAP when applied to freshly dissected rat sciatic nerves. Thus, in rats, potassium channels are absent from Ranvier nodes, in agreement with the results of previous workers¹²⁻¹⁵.

The second group of experiments (Fig. 2*b*) investigated the effect of 4-AP on nerves incubated in NR for longer times. After the first 20 min equilibration in NR, the nerve was left in the same solution for an additional 60 min. The upper tracing of Fig. 2*b* shows the CAP registered immediately after incubation in NR, while the lower tracing shows the CAP recorded after 15-min treatment with 4-AP. This experiment was performed 10 times and the results are summarized in Table 1. 4-AP seems to have no significant effect on CAP duration when applied to unstimulated nerves left in NR for 1 h.

Table 1 The effect of 4-aminopyridine on compound action potential of various nerve preparations

Group	No. of experiments	Effect of 4-AP on half-width (fraction of control)
<i>a</i> Freshly dissected unstimulated nerves	12	$1.038 \pm 0.047^*$
<i>b</i> 1 h in NR unstimulated nerves	10	$1.012 \pm 0.012^*$
<i>c</i> Nerves repetitively stimulated for 1 h at 200 Hz	10	$1.261 \pm 0.042^*$
t-test	<i>t</i>	<i>P</i>
<i>a</i> vs <i>b</i>	0.5002	NS
<i>a</i> vs <i>c</i>	3.4857	<0.002
<i>b</i> vs <i>c</i>	5.6474	<0.001

* Values represent $\pm$ s.e. NS, not significant.

The third group of experiments (Fig. 2*c*) shows the effect of 4-AP on nerves stimulated repetitively. After the initial 20 min in NR, the nerve was stimulated supramaximally at 200 Hz for 60 min. The continuous observation of the CAP in the oscilloscope allows one to verify that the nerve was actually transmitting impulses during the whole period of stimulation. Figure 2*c* (upper tracing) shows the CAP recorded from the repetitively stimulated nerve. After electrical stimulation, the nerve was perfused with 4-AP for 15 min and the CAP immediately recorded (Fig. 2*c*, lower tracing). Comparison between the two tracings of Fig. 2*c* shows that, in conditions of nerve activity, 4-AP produced a significant widening of the potential, similar to that observed by Chiu and Ritchie³. This experiment was repeated 10 times with similar results and the effect of 4-AP was apparent in all stimulated nerves, even though CAP widening varied between nerve specimens. The duration of the CAP at half-amplitude was 1.261 ± 0.042 (s.e.m.) times that of unstimulated controls (Table 1). The fact that, in rat sciatic nerves, the repetitive propagation of action potentials causes an acceleration in the rate of myelin swelling, concomitantly with the appearance of potassium channel activity, suggests a change in the permeability properties of the paranodal axoglial junctions, subsequent to nerve stimulation. The stability of the axoglial junctions is known to require the presence of an appropriate concentration of Ca^{2+} ions¹⁶; myelin swelling occurs sooner in nerves preincubated for 1 h in NR without divalent cations¹⁷. Therefore, we suggest that, as in nonmyelinated nerves¹⁷, a change in the ionic environment in the vicinity of active fibres occurs with increased impulse activity, producing structural changes in the paranodal region. The opening of the paranodal axoglial junctions by electrical stimulation may well be a physiologically significant phenomenon.

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Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones

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Oestrogen, acting in both the brain and pituitary, has a critical role in regulating the reproductive cycle in most mammals^{1,2}. In the brain, oestrogen regulates the release of luteinizing hormone-releasing hormone (LHRH) partly through a mechanism that is blocked by inhibitors of DNA-dependent RNA synthesis^{3,4} or protein synthesis⁴. The distributions of oestrogen-concentrating neurones⁵ and of LHRH neurones^{6,7} overlap. The present study was undertaken to determine whether genomic effects of oestrogen mediated by nuclear

oestradiol concentration include a direct effect on LHRH-containing neurones. During extensive studies in which the immunocytochemical method for localizing LHRH neurones was optimized and made compatible with the autoradiographic method for detecting oestrogen-concentrating neurones, doubly-labelled cells were very rarely seen. This suggests that genomic regulatory effects of oestrogen which depend on nuclear retention are not exerted directly on most LHRH neurones, but rather must be mediated by another class of neurones.

The methods used were modifications of those used successfully to identify neurophysin-containing neurones which concentrate oestrogen in their nuclei⁸. In preliminary studies directed at optimizing and combining the immunocytochemical and autoradiographic methods we used 24 female rats, 12 male rats, 3 female mice and 2 male mice⁹. The results reported here derive from oestrogen-concentrating and LHRH-containing neurones localized in the tissue sections taken from forebrain areas of three ovariectomized, colchicine-treated rats (50 µg per 2 µl injected into the lateral ventricle, 24 h before killing). We followed the established practice⁵ of using tissues from rats in which circulating oestrogens were reduced or eliminated (that is, rats were ovariectomized for 5 days) before injection of radiolabelled oestradiol, to avoid competition of 'cold' oestrogen with 'hot' oestrogen at receptor sites. Exchange assay data indicate that ovariectomized rats (5 days) receiving oestradiol injections or implants 2 h before being killed have at least as many nuclear oestrogen receptors in the brain as do pro-oestrous rats¹⁰.

Each rat received two intraperitoneal (i.p.) injections (to a total of 0.1–0.15 ml per 100 g body weight) of tritiated oestradiol [2, 4, 6, 7, 16, 17-3H(N), 151 Ci mmol⁻¹ (1 mCi in 1.8 µg oestradiol; 1 mCi ml⁻¹)-NEN] beginning 2.5 h before the rat was killed. One additional ovariectomized, colchicine-treated rat not receiving radioactive oestradiol was killed to provide tissue for controls. Animals were anaesthetized with pentobarbital, injected intracardially with 0.4 ml heparin and perfused with 0.025 M phosphate-buffered saline (PBS; pH 7.2, room temperature, 5 min) followed by Zamboni's buffered picric acid–paraformaldehyde fixative (pH 7.4, 25 min). Brains and pituitaries were post-fixed in Zamboni's for 24 h at 4 °C, immersed in 30% sucrose until sinking, and frozen onto brass chucks with dry ice. The tissue was cut (6 or 12 µm) in a cryostat at -20 °C in safelight conditions, then mounted directly onto emulsion-coated slides (Kodak NTB3).

Following exposure at -15 °C, the autoradiograms were developed as follows: slides were brought to room temperature in closed, desiccated boxes, air-dried in a desiccator for an additional 15 min, post-fixed for 30 s in Zamboni's and rinsed in two changes of PBS. Autoradiograms were developed in Kodak D-170 developer (7.5 min at 15 °C), stopped in distilled water (1 min at 15 °C) and fixed in Kodak fixer (5.5 min at 15 °C). The slides were rinsed twice in PBS, and primary antisera diluted in 0.2% Triton–PBS were applied. After incubation for 48 h at 4 °C in primary antisera, sections were processed by the indirect peroxidase–antiperoxidase method^{7,11}.

In preliminary studies, LHRH neurones were localized in 30-µm cryostat sections using four different antisera: Arimura 743 at 1:500; Silverman–Nilaver rabbit III at 1:500; Jackson 29 at 1:500; and Benoit LR1 at 1:10,000. The morphology and distribution of LHRH neurones in the forebrain was similar for all four antisera; however, the number and staining intensity of LHRH cell bodies and fibres were greatest using the Benoit LR1 antiserum. Our studies provided no evidence for a subclass of LHRH neurones capriciously revealed by a particular antiserum or experimental circumstance.

The number of LHRH neurones localized per brain with LR1 antiserum in optimal circumstances was almost 1,000. LR1 antiserum was raised in a rabbit against (D-Lys 6)-LHRH conjugated to ovalbumin with glutaraldehyde. The antigenic determinant consists of amino acids 2, 3, 4 and 7–10 of the decapeptide (R. Benoit, personal communication). Preabsorp-

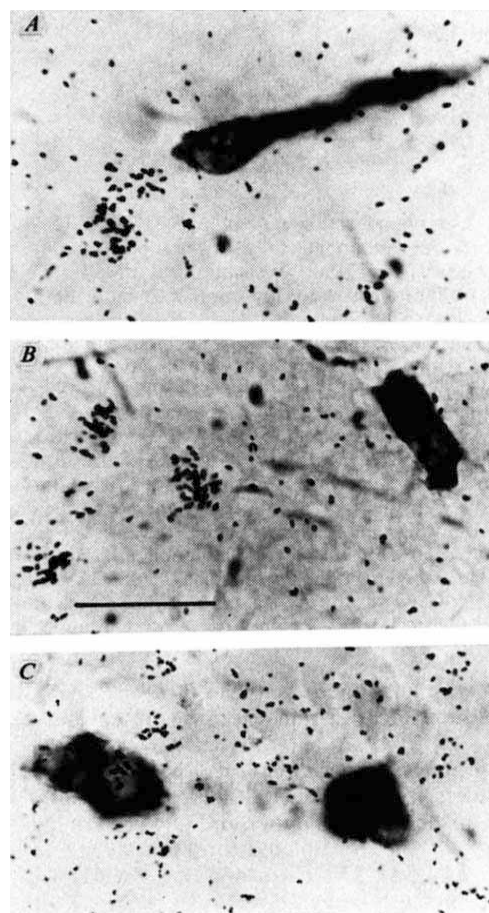


Fig. 1 A, B, Photomicrographs of LHRH neurones and oestradiol autoradiograms in the rat brain. Autoradiographic exposure time was 161 days. In immunocytochemically-localized LHRH neurones, dark reaction product is seen in the cytoplasm surrounding the relatively clear nuclei. Clusters of reduced silver grains occur in the emulsion underlying nuclei of oestrogen-concentrating cells, as determined by subsequent counterstaining with cresyl violet. The nuclei of LHRH neurones did not concentrate oestradiol. In A, an LHRH neurone is adjacent to two oestrogen-concentrating cells. In B, an LHRH neurone is located near three oestrogen-concentrating cells. C, Photomicrograph of immunocytochemically localized pituitary gonadotrophs and oestradiol autoradiograms. Autoradiographic exposure time was 168 days. Nuclei of both gonadotrophs concentrated radioactive oestradiol. Scale bar (in B), 20 µm.

tion of LR1 antiserum at 1:10,000 with 10 µm LHRH (Chemalog) for 24 h at 4 °C eliminated staining. For localizing luteinizing hormone (LH) in the pituitary, antiserum to β -LH at 1:1,000 was applied (National Hormone and Pituitary Program, Parlow).

A cell was designated as oestrogen-concentrating if there were at least five times as many reduced silver grains in the emulsion under the cell nucleus as there were grains under a similar-sized area of adjacent neuropil¹². Controls for chemography artefacts were negative. Autoradiograms were developed following exposure for 86, 118, 161, 168 or 245 days at -15 °C; most of the results derive from autoradiograms exposed for ≥ 161 days. Autoradiograms revealed oestrogen-concentrating neurones of similar number and distribution to those described previously for both unfixed⁵ and fixed¹³ tissue. Oestrogen-concentrating cells were counted in nine randomly-selected sections containing LHRH neurones; we found an average of 250 ± 70 ($\pm$ s.e.) oestrogen-concentrating cells per section.

The distribution of LHRH neurones was similar to that reported previously^{6,7}. LHRH neurones were localized in the

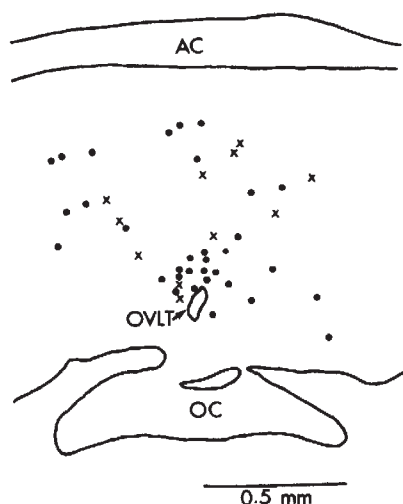


Fig. 2 Camera lucida drawing of autoradiographically localized oestradiol-concentrating neurones (●) and immunocytochemically localized LHRH neurones (×) in a 6- μ m coronal section from the brain of an ovariectomized, colchicine-treated rat. Autoradiographic exposure time was 161 days. None of the LHRH neurones concentrated radioactive oestradiol. These cells are in the rostral medial preoptic area: dorsal towards the top. AC, anterior commissure; OC, optic chiasm; OVLt, organum vasculosum lamina terminalis.

medial septum, diagonal bands of Broca, medial preoptic area, bed nucleus of the stria terminalis and the anterior hypothalamic area, especially dorsal to the supraoptic nuclei. LHRH neurones in the rostral fringe of the medial septum¹⁴ and nervus terminalis¹⁵ were not examined. None of the LHRH antisera used in the present study or in other studies^{6,14,16} supports the claim¹⁷ that a large population of LHRH neurones exists in the arcuate nuclei and median eminence of the rat, although occasionally we visualized LHRH neurones ventral and lateral to the ventromedial nuclei¹⁸. In any case, genomic actions of oestradiol on LHRH release (that is, "positive feedback"; refs 3, 4) are mediated in forebrain areas rostral to the mediobasal hypothalamus¹⁹.

Of 435 LHRH-immunoreactive cell nuclei examined in 240 sections (range 0–15 LHRH nuclei per section), radioactive oestradiol was retained in only one LHRH cell nucleus. Only those LHRH cell nuclei that were resolved in the plane of focus of the tissue section surface nearest the emulsion were included in the total. This served to decrease, but not eliminate, the contribution of LHRH cell nuclei which may have been oestrogen-concentrating, but too distant from the emulsion to have been detected autoradiographically. As few as 0.2% of LHRH neurones examined concentrated oestradiol, and a far smaller percentage of oestradiol-concentrating neurones produced LHRH. In many cases oestrogen-concentrating cells were identified a few micrometres away from LHRH cell nuclei (Fig. 1A, B and Fig. 2). The brain region with maximum intermingling of LHRH neurones and oestrogen-concentrating cells was dorsal and lateral to the organum vasculosum lamina terminalis (Fig. 2). In confirmation of previous work^{20,21} and to establish that the method described here could demonstrate oestrogen-concentrating cells producing particular peptides or proteins, oestrogen-concentrating pituitary gonadotrophs containing LH were identified (Fig. 1C).

The results reported here strongly suggest that nuclei of most LHRH neurones do not retain oestradiol to any appreciable extent. Given the prevailing concept that direct genomic effects of oestrogen depend on its retention by nuclear receptors^{22,23}, our results imply that genomic actions of oestrogen on LHRH release must be mediated by another class of neurones. The present results do not, however, exclude the possibility that oestrogen influences LHRH release through non-genomic mechanisms.

We thank Dr Joan King for supplying Arimura 743 and Jackson 29 LHRH antisera; Dr Ann-Judith Silverman for Silverman-Nilaver rabbit III LHRH antiserum; Dr R. Benoit for LR1 LHRH antiserum; and Dr Freja Kamel for the anti-LH β antiserum which she obtained from Dr A. F. Parlow through the NIAMDD Rat Pituitary Hormone Distribution Program.

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Adult and embryonic mouse neural cell adhesion molecules have different binding properties

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Interactions between neural cell surfaces seem to be of prime importance during neurogenesis, and responsible for the guidance of migrating neuroblasts and growing axons and for the formation of synapses. Little is known about the underlying molecular mechanisms, but most hypotheses imply the existence of cell-surface molecules that mediate the formation of transient or permanent bonds between neural cells. Recently, a membrane glycoprotein called neural cell adhesion molecule (N-CAM) has been characterized in chick^{1,2} and rodent³ nervous tissue that appears to act as a ligand in adhesion among neural cell bodies^{4,5} or neurites⁶. We have identified a mouse neural surface glycoprotein, named BSP-2 (ref. 7), which by criteria of electrophoretic migration^{7–9}, developmental changes^{8,9}, amino acid¹⁰ and sugar¹¹ composition seems to be closely related or identical to N-CAM. Both BSP-2 (refs 8, 9) and N-CAM^{2,12} undergo conversion from an embryonic to an adult form during brain development and it has been suggested that this transition changes the adhesive properties¹² or the binding specificity⁸ of the molecule. Using a neuroblastoma line to study functional differences between embryonic and adult BSP-2/N-CAM molecules, we show here that liposomes bearing adult BSP-2 but not those bearing the embryonic form adhere to neuroblastoma cells, demonstrating that the two forms do indeed possess different binding properties.

BSP-2 and N-CAM exist in several molecular weight forms which are selectively expressed in different nervous tissues⁸ and during development^{2,8,9,12}. BSP-2 purified from embryonic

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mouse brain migrates as a broad zone in the 190,000–250,000 (190K–250K) molecular weight region of SDS-polyacrylamide gels. By contrast, three components of 180K, 140K and 120K are characteristic of mature nervous tissues. Available evidence indicates that these different forms are due to glycosylation differences of identical polypeptide chains^{2,8}. A form of BSP-2 resembling the adult antigen is also expressed by cells of the N1E 115 neuroblastoma clone⁸. All cells in N1E 115 cultures fluoresced brightly when stained with anti-BSP-2 monoclonal antibodies (not shown). Furthermore, these cells appeared to have a BSP-2-dependent adhesion mechanism, as their rate of aggregation was strongly inhibited by Fab' fragments of rabbit anti-BSP-2 antibodies, but not by antibodies directed against an unrelated surface protein (Fig. 1). These data suggested that the N1E 115 clone could represent a useful model system to investigate further the adhesive properties of BSP-2 molecules.

If one of the molecular species of BSP-2 were to be a ligand in the formation of cell-cell bonds, the purified molecules should bind to a cell-surface receptor. To test this, embryonic and adult BSP-2 purified in octyl glucoside from whole mouse brain were reinserted in lipid vesicles made from phosphatidylcholine and cholesterol and containing 40 mM carboxyfluorescein label. Around 50% of the CF content of liposomes bearing either the embryonic or the adult species could be specifically immunoprecipitated by anti-BSP-2 monoclonal antibodies which recognize the antigen at the surface of intact cells⁷. This result demonstrates that part of the liposomes displayed the molecule at their external surface in a rightside-out orientation.

Binding of liposomes bearing the adult form of BSP-2 to N1E 115 cells was easily visualized by fluorescence microscopy. Cells incubated with these lipid vesicles showed bright surface fluorescence distributed in patches (Fig. 2a). Perinuclear fluorescence in some cells seemed to indicate endocytosis of the vesicles, but this was seen in only a few cells. Cells incubated with control lipid vesicles not containing protein were labelled only very weakly (Fig. 2e). We then investigated whether the embryonic form of BSP-2 also mediated binding of liposomes to N1E 115 cells. As shown in Fig. 2c, cell-associated fluores-

cence after incubation with liposomes containing embryonic BSP-2 was very faint and not stronger than that seen with liposomes containing only lipids. We conclude from these experiments that during maturation of the nervous system, BSP-2 molecules acquire adhesion properties which enable them to bind to N1E 115 cells. Although these data show that BSP-2 purified from adult brain has binding affinity for N1E 115 cells, it provides no evidence that the binding is involved in the

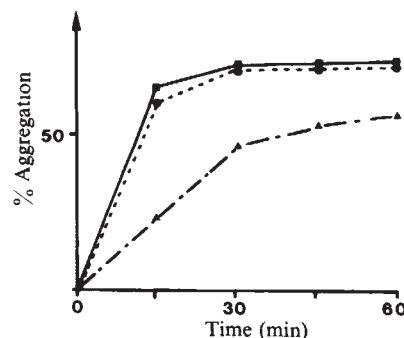
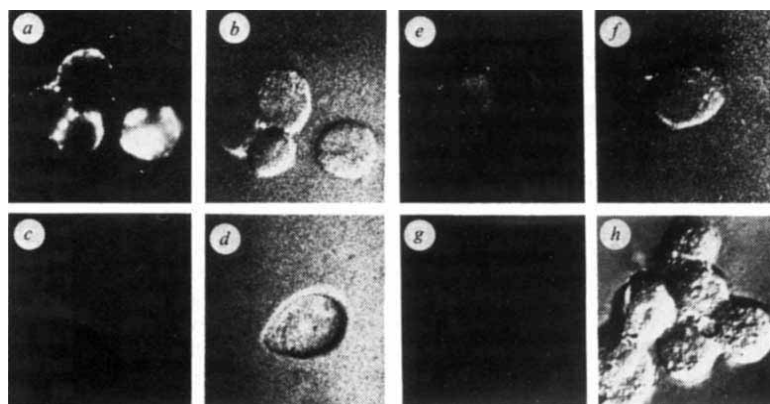


Fig. 1 Inhibition of aggregation of N1E 115 neuroblastoma cells by rabbit anti-BSP-2 antibody. Per cent aggregation is plotted as a function of time in the absence of added antibody (—■—) or in the presence of 0.25 mg ml⁻¹ of Fab' fragments of rabbit anti-BSP-2 (---▲---) or rabbit anti-BSP-1 (---●---) IgG. N1E 115 cells^{14,15} were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. They were detached with Earle's salt solution (Ca²⁺- and Mg²⁺-free) containing 1 mM EDTA, washed once and resuspended in the same solution at 1.5 × 10⁶ ml⁻¹. At this stage, the cells were present as single cells >95% viable. Aggregation was measured by determining the rate of decrease of single cells. 2 ml of the cell suspension were shaken (100 r.p.m., 37 °C) in round-bottomed tubes precoated with fetal calf serum. Aliquots were taken at various times and the cells present singly or as doublets counted manually. Rabbit antibodies to BSP-2 were produced by three injections of material affinity-purified from adult mouse brain. Anti-BSP-1 antibodies are directed against an unrelated 140K surface glycoprotein¹⁶, also expressed by N1E 115 cells.

Fig. 2 Binding of fluorescent liposomes bearing adult (a) or embryonic (c) forms of BSP-2 or containing only lipids (e) to N1E 115 neuroblastoma cells. g Shows the inhibition of binding of liposomes containing adult BSP-2 by rabbit anti-BSP-2 antibody. a, c, e, g, Fluorescence micrographs; b, d, f, h, Nomarski DIC, of the same field (×850).

Methods: BSP-2 was purified from adult or embryonic (day 17–19) mouse brain membranes⁷. Membranes were extracted in 1% Triton X-100 in 20 mM Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl and the protease and neuraminidase inhibitors used previously⁹. The extracts were passed through two columns operated in tandem, the first containing rat IgG coupled to Sepharose, the second Sepharose-bound anti-BSP-2 monoclonal antibody⁸. After washes with 20 mM Tris buffer (pH 7.6) and borate (0.1 M)/NaCl (1 M) buffer (pH 8.5), both containing 0.2% Triton X-100, the detergent was changed to octyl β-D-glucopyranoside. Bound antigen was eluted from the monoclonal antibody column with 50 mM diethylamine (pH 11.5)/34 mM octyl glucoside. After neutralization with solid glycine, the eluted material was dialysed against 34 mM octyl glucoside in 0.15 M NaCl/50 mM Tris-HCl (pH 7.4) (Tris/saline). The purity of the purified antigen was comparable with that observed after purification in deoxycholate⁸. For reconstitution into lipid vesicles, 300 μg of purified BSP-2 were mixed with 2.3 μmol egg yolk phosphatidylcholine and 1.2 μmol cholesterol in half-strength Tris/saline containing 91 mM octyl glucoside and 40 mM CF. After 2 h at 11 °C, the detergent was removed by chromatography on a Sephadex-G25 (27 ml) column, of which 11 ml were occupied by 40 mM CF, followed by dialysis against Tris/saline. Liposomes were separated from unbound protein by sucrose gradient centrifugation¹⁷ and dialysed against 0.15 M NaCl/20 mM HEPES (pH 7.2). Protein incorporation was monitored by adding trace amounts of radioiodinated BSP-2, and was 54–78%. Initial quenching ratios¹⁸ for CF fluorescence were 84–92%. Some leakage of CF occurred during storage of the liposomes at 4 °C, as indicated by decreased quenching, but did not, however, affect their binding properties and the background fluorescence, as the same results were obtained with vesicle preparations with ratios of 60–92%. N1E 115 cells grown as monolayers were detached by pipetting and made to adhere to polylysine-coated glass coverslips. After 1 h at 37 °C, 100 μl of a liposome suspension diluted to a CF concentration of 2.5 μM in DMEM containing 10 mg ml⁻¹ bovine serum albumin was added and left for 1 h at room temperature. The coverslips were then washed and mounted in Elvanol. To test for inhibition of binding by antibody, liposomes containing adult BSP-2 were diluted to 2.5 μM CF in DMEM and incubated for 3 h at 4 °C in the presence of rabbit anti-BSP-2 Fab'. The suspension was then used to label N1E 115 cells. Quenching ratios did not change during incubation in the presence of antibody, and incubation with irrelevant antibody did not reduce staining by liposomes (not shown).



formation of cell-cell bonds. However, this is made likely by our observation that the monovalent rabbit anti-BSP-2 antibodies which inhibit aggregate formation also inhibited the fixation of adult BSP-2 to the surface membrane (Fig. 2h).

The specificity of the BSP-2-mediated interaction of liposomes with neural cells was further demonstrated by the use as targets of cultured astrocytes, which lack BSP-2 antigen^{7,10} (Fig. 3b). Neither embryonic nor adult BSP-2 seemed to adhere to these cells. Staining by liposomes containing either form of BSP-2 was very faint and not different from that seen with pure lipid vesicles. This result supports the proposal⁵ that neural cell adhesion molecules bind to each other. However, we have no direct evidence that BSP-2 forms complexes with itself.

Embryonic N-CAM^{2,3} and BSP-2¹¹ have a threefold higher sialic acid content than the adult molecules. The negative charge contributed by the sialic acid residues could reduce the binding strength of the embryonic form through electrostatic repulsion.

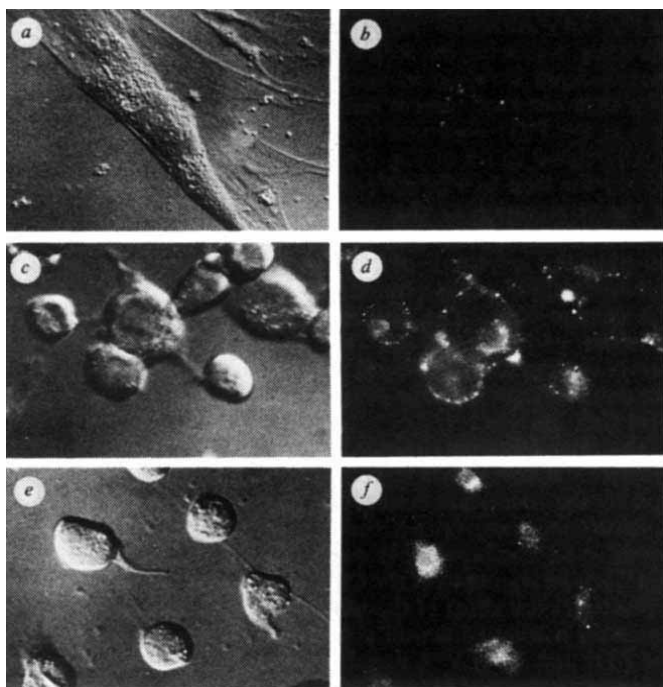


Fig. 3 Lack of binding of BSP-2-bearing liposomes to cultured astrocytes (a, b) and effect of neuraminidase treatment on the binding of embryonic BSP-2-containing liposomes to N1E 115 cells (c-f). a, b, Labelling of astrocyte cultures with liposomes bearing adult BSP-2 was done as described in Fig. 2 legend. Fluorescent spots around the nucleus are due to reddish autofluorescence. Surface fluorescence is hardly detectable. c-f, Surface staining of N1E 115 cells by embryonic BSP-2 reconstituted into lipid vesicles is clearly seen after incubation with neuraminidase-digested liposomes (d), but absent when neuraminidase was inhibited (f). a, c, e, Nomarski DIC; b, d, f, fluorescence micrographs of the same field ($\times 1,000$).

Methods: Astrocyte cultures were prepared essentially as described elsewhere¹⁹. Newborn mouse cerebral cortex was dissociated using trypsin and collagenase. Non-astrocytic cells were removed by vigorous shaking of the culture dishes, followed by treatment with cytosine arabinoside. The cultures used in these experiments contained >95% glial fibrillary acidic protein-positive astrocytes. After 14 days *in vitro*, the cells were removed from the dishes by trypsinization, plated on polylysine-coated coverslips and grown for a further 2 days before use. To test the effect of neuraminidase treatment, a liposome suspension containing embryonic BSP-2 was made 1 mM in CaCl_2 and incubated in the presence of 50 U ml⁻¹ neuraminidase from *Vibrio cholerae* (Hoechst-Behring) for 5 h at 37 °C. The reaction was stopped by adding 2.8 mM of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, a specific neuraminidase inhibitor²⁰. Another aliquot of the same preparation was treated similarly except that the inhibitor was added together with neuraminidase.

Other explanations, however, such as the masking of sugars by terminal sialic acid or conformational differences induced by changes in the carbohydrate structure, have not been excluded. In agreement with a role for sialic acid in the adhesive properties of BSP-2, we observed increased cell-associated fluorescence after neuraminidase treatment of liposomes containing embryonic (Fig. 3c-f) but not adult (not shown) BSP-2. This result also demonstrates that absence of labelling with the embryonic form is not due to preferential denaturation of this species.

Our results seem to contradict previous reports on the binding of lipid vesicles containing embryonic chick N-CAM to dissociated brain and muscle cells from chick embryos^{4,13}. Species differences could account for this disparity, but it is more likely that the binding specificity of N1E 115 cells differs from that of embryonic brain cells. Embryonic BSP-2/N-CAM may still be able to bind to N1E 115 cells, but with too low an affinity to be detected by our methods. This possibility is difficult to discount because of the inability to determine affinity parameters for ligands reconstituted into liposomes. We routinely incubated the liposomes with cells attached to glass coverslips for 1 h at room temperature. However, even labelling N1E 115 cells in suspension for 15 min at 37 °C, as did Rutishauser *et al.*⁵ using chick brain cells, revealed no binding of embryonic BSP-2. In fact, if anything, the labelling mediated by the adult form was less intense in these conditions. In our hands, freshly dissociated³ embryonic mouse brain cells bound BSP-2-containing liposomes poorly. Trypsinization may partially degrade surface receptors for BSP-2 or residual trypsin released from the cells may attack BSP-2 in liposomes. In any case, neuroblastomas, which do not require dissociation, represent an interesting model for studying the binding of cell adhesion molecules to surface receptors on neural cells. Our data further show that adhesion molecules present in normal tissue are involved in the adhesive interactions of tumour cells.

The present findings provide direct evidence that the developmental changes in the structure of neural cell adhesion molecules are correlated with differences in their binding characteristics. The structural changes seem to be created by different glycosylation of a common polypeptide backbone^{2,8}. It seems, then, that neural cells may acquire adhesion competence or new binding specificities by modifying the carbohydrate moieties of surface glycoproteins.

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Detection of intracellular Ca^{2+} transients in sympathetic neurones using arsenazo III

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Changes in cytosolic calcium ion concentration ($[\text{Ca}^{2+}]_i$) have been implicated in a wide variety of cellular stimulus-transduction roles¹⁻³. In nerve cells, it is believed that electrical activity raises $[\text{Ca}^{2+}]_i$ by allowing influx of Ca^{2+} through voltage-dependent channels in the surface membrane^{4,5}. Elevation of neuronal $[\text{Ca}^{2+}]_i$ may also occur due to release of Ca^{2+} from intracellular storage sites⁶⁻⁸. Transient increases in $[\text{Ca}^{2+}]_i$ are thought to trigger neurotransmitter release⁹, and to modulate axonal transport¹⁰, energy metabolism¹¹ and growth cone movement¹². Intracellular Ca^{2+} also appears to regulate membrane potassium channels and thereby to regulate electrical excitability¹³. Although $[\text{Ca}^{2+}]_i$ transients have been measured in a few giant invertebrate neurones¹⁴, detection of such transients in a vertebrate neurone has not been previously reported. We have measured $[\text{Ca}^{2+}]_i$ in bullfrog sympathetic neurones by photometry of a microinjected calcium indicator dye, arsenazo III (refs 14-16), and report here that action potentials and voltage-clamped depolarizations cause long-lasting increases in $[\text{Ca}^{2+}]_i$. Also, exposure to the drug theophylline can cause spontaneous periodic increases in $[\text{Ca}^{2+}]_i$. Comparisons of $[\text{Ca}^{2+}]_i$ signals with simultaneous intracellular recordings of membrane potential suggest that the kinetics of the post-tetanic hyperpolarization (PTH) following a series of action potentials or the spontaneous hyperpolarizations induced by theophylline directly reflect the kinetics of the $[\text{Ca}^{2+}]_i$ transient.

In many neurones, a PTH occurs following a train of action potentials. In bullfrog sympathetic neurones, the PTH is thought to be generated by a Ca^{2+} -sensitive K^+ current activated by Ca^{2+} entry during the series of action potentials^{17,18}. It is not clear, however, whether the long duration of the PTH in these neurones is due to a prolonged elevation of $[\text{Ca}^{2+}]_i$ or to slow kinetics of the Ca^{2+} -sensitive K^+ conductance. To study the alteration of $[\text{Ca}^{2+}]_i$ associated with membrane electrical activity, we injected the calcium indicator dye, arsenazo III, into bullfrog sympathetic neurones and monitored the optical absorbance of individual cells by microphotometry (see Fig. 1 legend). Figure 1 illustrates an experiment designed to study the relationship between the membrane potential change following a train of action potentials (Fig. 1A) and the arsenazo III signal (Fig. 1B). In the intracellular voltage recording of Fig. 1A, the PTH can be seen after the termination of stimulation. The four lower traces in Fig. 1B show the simultaneous recording of light transmittance at four separate wavelengths: 570, 610, 660 and 700 nm. The top trace is the difference between the 700- and 660-nm signals; this differential signal was used as the indicator of $[\text{Ca}^{2+}]_i$ (see Fig. 1 legend). Figure 1B shows that the $[\text{Ca}^{2+}]_i$ signal increased progressively during the series of action potentials and began a gradual return to baseline at the end of the action potential train. The recovery of the $[\text{Ca}^{2+}]_i$ signal followed a time course that was as long as or longer than the repolarization of the PTH. The progressive increase in the $[\text{Ca}^{2+}]_i$ signal during the train of repetitive action potentials presumably reflects a summation of $[\text{Ca}^{2+}]_i$ that entered during each action potential. If both the PTH and the $[\text{Ca}^{2+}]_i$ signal result from the entry of Ca^{2+} , inhibition of Ca^{2+} influx would be expected to block both responses. Figure 2

shows that both the $[\text{Ca}^{2+}]_i$ signal and the PTH were reversibly abolished by the addition of 5 mM Mn^{2+} to the Ringer's solution; manganese is known to inhibit Ca^{2+} entry in many cell types^{4,5}.

A voltage-dependent Ca^{2+} current which might give rise to the observed $[\text{Ca}^{2+}]_i$ signal has been described in voltage-clamp experiments on bullfrog sympathetic neurones¹⁹. This Ca^{2+} current has a bell-shaped dependence of current magnitude on membrane potential, with the peak current observed near +10 mV. If both the $[\text{Ca}^{2+}]_i$ signal and the Ca^{2+} -sensitive K^+ conductance underlying the PTH are graded consequences of this voltage-dependent Ca^{2+} current, they should exhibit a similar bell-shaped dependence on pulse voltage. Figure 3 illustrates an experiment designed to test this prediction. In these voltage-clamp experiments, pulses to various membrane potentials were applied and both the membrane current response and the optical $[\text{Ca}^{2+}]_i$ signal were recorded. In the experiment illustrated in Fig. 3A, the membrane was held at a potential of -35 mV and stepped to +20 mV for 1 s (trace 1 of Fig. 3A). Although the current response to the step was off the scale, following the step there was an outward tail current that decayed slowly over several seconds (trace of Fig. 3A). This slow outward current tail probably reflects the same process as the PTH observed in current-clamp experiments. The $[\text{Ca}^{2+}]_i$ signal (trace of Fig. 3A) showed a progressive increase during the step to +20 mV; decay of the $[\text{Ca}^{2+}]_i$ signal began at the termination of the voltage pulse and followed a slow time course similar to that of the outward tail current. Figure 3B plots the amplitude of both the $[\text{Ca}^{2+}]_i$ signal and the slow outward tail current as a function of membrane potential during the voltage pulse. The two curves are bell-shaped and peak between +10 and +20 mV. The shape and peak of the two curves suggest that both the $[\text{Ca}^{2+}]_i$ signal and the slow outward tail current result from the previously described voltage-dependent Ca^{2+} current.

The after-hyperpolarization following an action potential in sympathetic neurones is increased in both amplitude and duration by the methylxanthines, theophylline¹⁸ and caffeine²⁰. It is uncertain, however, whether the potentiation of this Ca^{2+} -sensitive K^+ conductance is due to an augmented elevation of $[\text{Ca}^{2+}]_i$ or an alteration of the Ca^{2+} -activated K^+ channel. Figure 4 illustrates the effect of theophylline on the hyperpolarization following a train of action potentials. Figure 4A shows both the PTH and the $[\text{Ca}^{2+}]_i$ signal associated with a train of action potentials in normal Ringer's solution. After theophylline addition, the amplitude and duration of the PTH increased (Fig. 1B, bottom trace). The $[\text{Ca}^{2+}]_i$ signal was similarly increased in both amplitude and duration (Fig. 1B, top trace). Theophylline and caffeine can also induce spontaneous rhythmic hyperpolarizations which involve a Ca^{2+} -sensitive K^+ conductance^{7,20}. Pharmacological observations suggest that this Ca^{2+} -sensitive K^+ conductance results from an intracellular release of Ca^{2+} (refs 7, 8). Figure 4B shows the $[\text{Ca}^{2+}]_i$ signal recorded simultaneously with spontaneous rhythmic hyperpolarizations (arrows) induced by theophylline. The arsenazo signal indicates that there was indeed an increase in $[\text{Ca}^{2+}]_i$ associated with the spontaneous hyperpolarizations. Moreover, the time course of the increased $[\text{Ca}^{2+}]_i$ seems similar to that of the spontaneous hyperpolarizations.

Although the spatial distribution of the Ca^{2+} transient is not resolved by this optical technique (see Fig. 1 legend), the measurement of the time course of $[\text{Ca}^{2+}]_i$ makes it possible to calculate the redistribution of Ca^{2+} expected from diffusion in the cytoplasm. Assuming no membrane barriers to diffusion, Ca^{2+} movement from the cell surface into the cytoplasm may resemble the mathematically simple case of diffusion from a plane source into a semi-infinite volume. In this regime, concentration falls to $1/e$ of the value near the source plane at a distance of $(4Dt)^{1/2}$ from that plane, where t is the time after instantaneous release of the diffusing substance and D is the diffusion coefficient²¹. Taking a value for D of $10^{-7} \text{ cm}^2 \text{ s}^{-1}$, an approximate value for the diffusion of Ca^{2+} in the cytoplasm

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Fig. 1 Action potential firing raises $[Ca^{2+}]_i$ in a bullfrog sympathetic neurone. The calcium indicator dye, arsenazo III, was injected into the cell before recording.

A, Membrane potential. Action potentials were elicited by antidromic stimulation of the postganglionic axon at a frequency of 30 Hz for 5 s. The period of stimulation is indicated by the bar labelled S. The action potentials are not observed because of high gain which puts the trace off-scale and limited pen recorder frequency response. Note the PTH, shown as the downward deflection, which persists for ~ 10 s after the stimulus train. **B**, Optical absorbance signals recorded simultaneously with the data shown in **A**. Light from a quartz-halogen source was focused on the cell using a microscope condenser and collected by an optical fibre positioned over the cell body. The intensity of the light collected by the optical fibre was measured simultaneously over four spectral bands by dispersing the light with a diffraction grating onto four silicon photodiodes. The photodiodes were sensitive to spectral bands 30 nm wide centred at 570, 610, 660 and 700 nm (wavelengths chosen to discriminate between the Ca^{2+} -induced absorbance change of arsenazo III and possible interference due to movement of the cell or change in pH or Mg^{2+}). The top trace is a differential transmittance signal obtained by subtracting transmittance at 660 nm from that at 700 nm. This signal detects optical absorbance changes due to arsenazo III binding of Ca^{2+} , and gives an upward deflection for an increase in Ca^{2+} concentration. The optical recording indicates that $[Ca^{2+}]_i$ increased progressively during action potential firing and recovered slowly towards the baseline after the stimulus train. The four lower traces in **B** show transmittance signals for the four individual wavelength bands. Transmittance (T) is specified as per cent of the pre-stimulus baseline at each wavelength. An optical signal for cell movement would be maximal at 570 nm, where the absorbance of the dye is greatest, while a pH change would give a maximal signal at 610 nm, with a much smaller change at 660 nm (ref. 24). The peak absorbance changes observed here were greater in amplitude at 660 nm than at 610 nm, which is the case for an elevation of Ca^{2+} . The rapid fluctuations in single-band transmittance signals reflect small movements of the cell; these fluctuations are reduced in amplitude by the differential signal processing. Intracellular dye concentration was estimated by measuring optical density at 570 nm before and after dye injection, and by visually measuring cell body diameter to calculate the optical path length. A correction for instrumental stray light was made by measuring asymptotic absorbance during overfilling of cells at the end of the experiments. For this cell, the path length was estimated at 60 μ m and the dye concentration was estimated to be 200 μ M. Using these data and calibration data obtained with ionic conditions similar to those of cytoplasm²⁵, the optical absorbance change shown is estimated as a change in $[Ca^{2+}]_i$ of 1.6 μ M averaged over the entire cell volume. As 150 action potentials were fired during the stimulus train, the average increment in $[Ca^{2+}]_i$ per action potential would be ~ 10 nM. For intracellular conditions, there are two major problems associated with quantitative estimates of Ca^{2+} concentration. First, complexation of Ca^{2+} with the dye is influenced by environmental factors such as pH, Mg^{2+} , ionic strength^{25,26} and the presence of certain proteins²⁷. Uncertainty regarding such cytoplasmic conditions therefore limits the accuracy of dye calibration. Second, as the optimal signal is recorded from the entire cell, any spatial gradient of Ca^{2+} within the neurone would be unresolved. In this regard, it is presumed that Ca^{2+} concentration reaches higher peak values near sites of Ca^{2+} influx^{28,29}. The experiments were performed on the ninth or tenth parasympathetic ganglion of the bullfrog, *Rana catesbeiana*. The ganglion was excised and placed in a recording chamber containing Ringer's solution. Type B cells (40–80 μ m diameter) were viewed under high power microscope optics ($\times 300$) and impaled with a micropipette filled with 100 mM KCl and 80 mM arsenazo III (Na^+ salt, grade I; Sigma); this electrode was used to record membrane potential and to inject arsenazo III. For voltage-clamp experiments, a second micropipette filled with 3 M KCl was used to pass current.

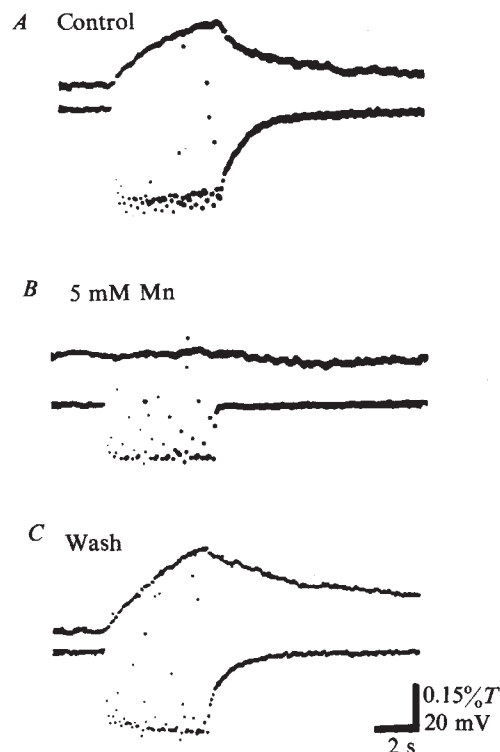
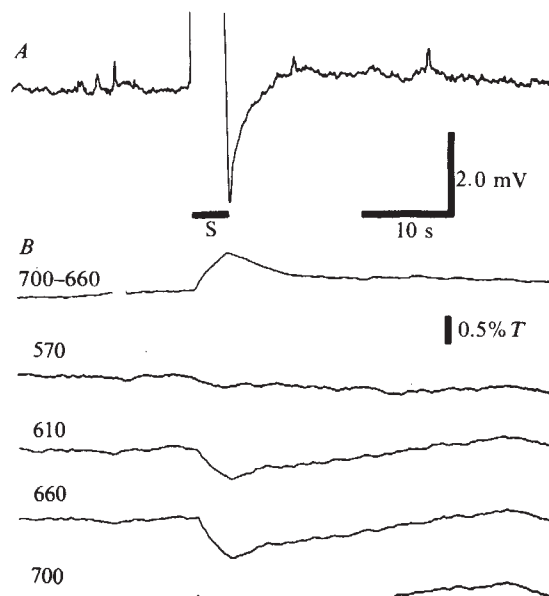


Fig. 2 Manganese ions (Mn^{2+}) added to the bathing medium reversibly abolish both the optical intracellular calcium signal, $[Ca^{2+}]_i$, and the PTH elicited by repetitive action potentials. Since extracellular Mn^{2+} is known to block the entry of Ca^{2+} through membrane Ca^{2+} channels in other cell types^{4,5}, this result suggests that both the optical signal and the PTH require membrane Ca^{2+} influx. For each pair of records, the upper trace is the optical $[Ca^{2+}]_i$ signal (700–660 nm transmittance difference) from an arsenazo-filled cell and the lower trace is the simultaneous record of membrane potential. The cell was stimulated antidromically at a frequency of 30 Hz for 4 s. The data were stored in digital form using a PDP 11/03 microcomputer; the scattered dots during the period of stimulation reflect the slow sampling of the rapid repetitive action potentials. **A**, Control Ca^{2+} signal and PTH in normal Ringer's solution; **B**, 7.5 min after addition of 5 mM $MnCl_2$ to the Ringer's solution. Note that the increase in $[Ca^{2+}]_i$ evident in **A** was eliminated, as was the PTH. **C**, 9 min after beginning Mn^{2+} washout. Note that the optical $[Ca^{2+}]_i$ signal has recovered and actually exceeds the amplitude of the control. The increase in signal amplitude presumably reflects leakage of additional dye from the micropipette into the cell during the time interval between records **A** and **C** (17 min).

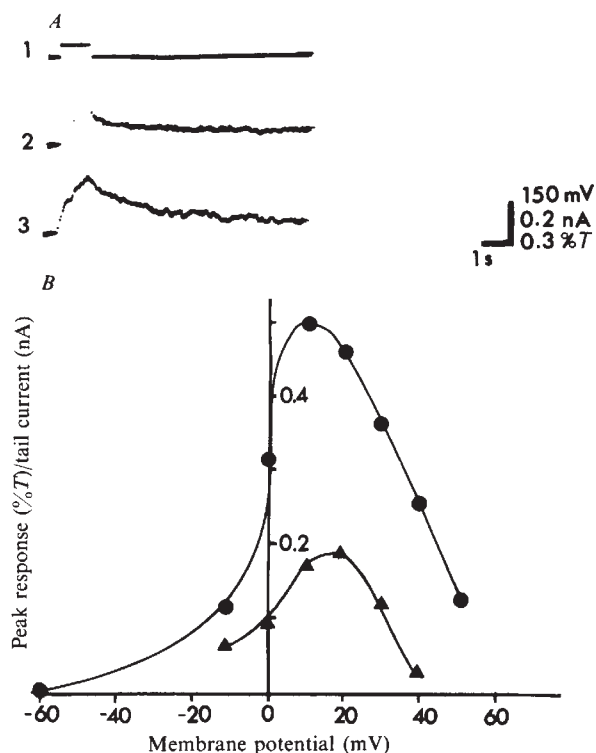


Fig. 3 Intracellular Ca^{2+} signal and outward tail current show a similar voltage dependence. **A**, Traces show: (1) membrane potential; (2) membrane current; and (3) optical calcium signal, $T_{700} - T_{660}$. The potential during the 1-s pulse was +20 mV. At the gain used to show the slow tail current, membrane current during the pulse was off the scale. Note that both the membrane current and the optical Ca^{2+} signal decay slowly after the pulse. **B**, Amplitude of the peak optical Ca^{2+} signal (●) and the outward tail current (▲) measured 250 ms after the end of the pulse, as a function of pulse potential. Note that both the optical Ca^{2+} signal and the tail current exhibit a maximum at a membrane voltage between +10 and +20 mV. The arsenazo-filled cell was voltage-clamped by the two-microelectrode method³⁰. Tetraethylammonium chloride (10 mM) was present in the bathing medium to facilitate voltage control during large depolarizations. Depolarizing pulses were 1 s in duration; membrane holding potential was -35 mV. This holding potential was used to enhance the driving force of the slow outward tail current, which appears to be carried by K^+ .

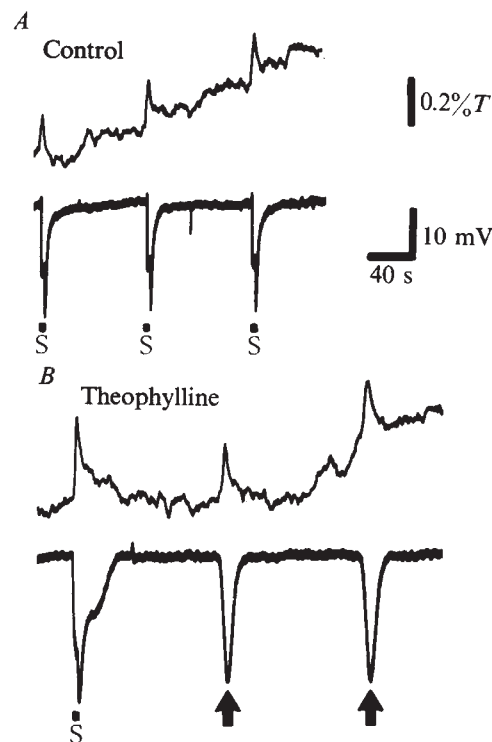


Fig. 4 Intracellular Ca^{2+} signals during PTHs and spontaneous rhythmic hyperpolarizations induced by theophylline. **A**, Optical $[\text{Ca}^{2+}]_i$ signal (top trace) and membrane voltage (bottom trace) in normal Ringer's solution. Record shows PTHs elicited by antidromic stimulation at a frequency of 30 Hz for 8 s; stimulation indicated by bars labelled S. The optical signal indicates an increase in $[\text{Ca}^{2+}]_i$ corresponding in time with the PTH. **B**, 8.3 min after addition of 20 mM theophylline to the Ringer's solution. Note that in theophylline the PTH and the corresponding $[\text{Ca}^{2+}]_i$ signal increased in both amplitude and duration. Following the antidromic stimulation to elicit the PTH (bar labelled S), two spontaneous hyperpolarizations occurred (arrows). The optical signals indicate increases in $[\text{Ca}^{2+}]_i$ simultaneous with the spontaneous hyperpolarizations.

of other cell types^{22,23}, it can be calculated that Ca^{2+} would diffuse $\sim 30 \mu\text{m}$ from a plane source in 10 s. As the calcium transients measured here last for over 10 s, it is reasonable to suppose that a series of action potentials can elevate $[\text{Ca}^{2+}]_i$ throughout the cell body of a neurone of $60 \mu\text{m}$ diameter.

The experiments reported here show that in a vertebrate neurone arsenazo III can detect increases of $[\text{Ca}^{2+}]_i$ resulting from membrane electrical activity, and periodic elevations of $[\text{Ca}^{2+}]_i$ induced by theophylline. This substantiates previous inferences from electrophysiological and pharmacological observations^{7,8,17,18}. The similar time course of the $[\text{Ca}^{2+}]_i$

transients and the membrane potential change implies that the kinetics of the membrane hyperpolarizations reflect the kinetics of the $[\text{Ca}^{2+}]_i$ transients. In addition, the slow kinetics of the $[\text{Ca}^{2+}]_i$ transients may have other important implications. As a consequence of its prolonged elevation, $[\text{Ca}^{2+}]_i$ would be expected to diffuse throughout the cell body and may thus affect a variety of Ca^{2+} -dependent biochemical processes such as the regulation of cytoskeletal structure, energy metabolism, axonal transport and gene expression. Moreover, as Ca^{2+} -sensitive molecular processes would be activated for the duration of the increase in $[\text{Ca}^{2+}]_i$, significant biochemical changes could result from long-lasting $[\text{Ca}^{2+}]_i$ transients. In this way, brief periods of membrane excitation might be translated into long-lasting changes in neuronal function.

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Two monoclonal antibodies against different antigens using the same V_H germ-line gene

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Immunoglobulin diversity seems to arise largely by three mechanisms: (1) the existence of several germ-line genes, which must be rearranged before expression—that is, V and J for the light (L) chains¹⁻³, V , D and J for the heavy (H) chains⁴; (2) somatic events, including mutations and gene conversion⁵⁻⁸; and (3) combinatorial association of heavy and light chains⁹⁻¹³, leading to the proposal that random pairing of $p \times H$ and $q \times L$ chains might generate $p \times q$ antibody molecules expressing discrete specificities. As heavy and light chains derived from the same immunoglobulin molecule would frequently reassociate preferentially¹⁴⁻¹⁷, it is likely that only a fraction of potential heavy-light pairs actually provides 'valid' antibodies. As a consequence of combinatorial heavy-light chain pairing, antibodies of discrete specificities sharing the same V_H region, associated with distinct light chains (or vice versa) should be encountered. We report here that two heavy chains, derived from the same V_H germ-line gene, may be present in anti-NP or anti-GAT antibodies, depending on their association with a specific λ or κ light chain, respectively.

A hybridoma was derived from a (C57BL/6 $\times$ DBA/2) F₁ mouse immunized with the GAT random copolymer, using the myeloma cell line NS1 for fusion^{18,19}. The corresponding monoclonal antibody was purified on a GAT-Sepharose column, and was found to express the common idiotype specificity, CGAT^{18,19}. It has been shown in a similar system that this specificity requires the presence of both the heavy and the light chains in order to be expressed²⁰. This monoclonal antibody was of the IgG1 class, and expressed the Igh b allotype of the C57BL/6 strain (data not shown).

Table 1 Association of V_H C.GAT/NP^b with V_K CGAT, V_K GA.1 or $V_{\lambda 1}$ chains derived from limited pools of germ-line genes produced discrete antibody specificities and idiotypes

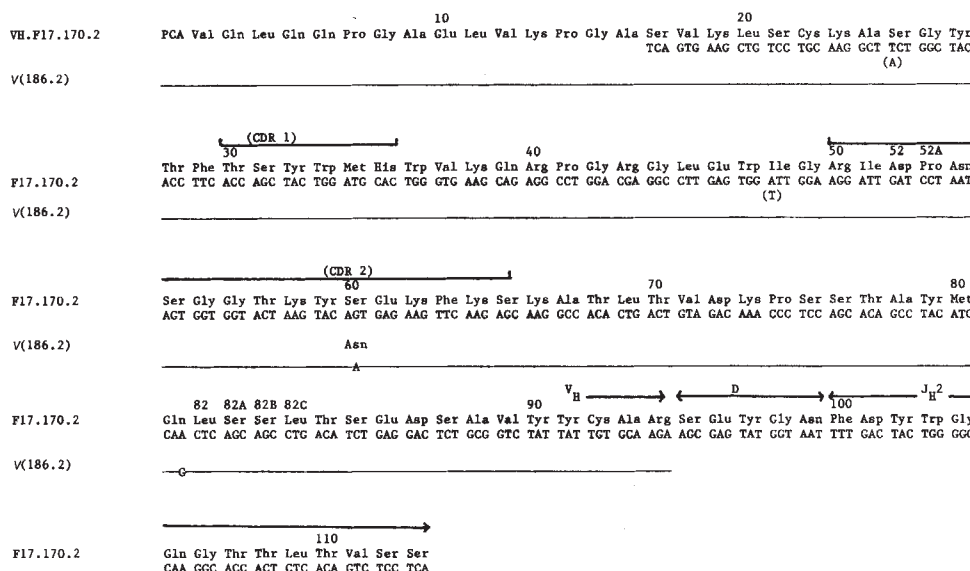
Germ-line genes V_H	V_L	V_K C.GAT (BALB/c or DBA/2)	V_K GA.1 (DBA/2)	$V_{\lambda 1}$ (C57BL/6)
V_H C.GAT (BALB/c or DBA/2)		Anti-GT* (C.GAT idio type)	Anti-GA (GA.idio type)	?
V_H NP ^b (C57BL/6)		Anti-GT* (C.GAT idio type)	?	Anti-NP (NP ^b idio type)

* Anti-GAT antibody that expressed the C.GAT common idio type recognized primarily the GT determinants.

The heavy-chain sequence data presented in Fig. 1 were obtained by NH₂-terminal amino acid automatic sequencing (residues 1–36), and by nucleotide sequencing (from codon 17), using the mRNA as a template for the dideoxy method adapted from Sanger^{21,22}. Identity of the protein and nucleic acid sequence within the overlapping stretch (amino acids 17–36, including the first hypervariable region) indicated that the unique nucleotide sequence that was determined indeed represented the mRNA actually encoding the anti-GAT heavy chain. The complete amino acid sequence was thus derived from residue 1 to 120, encompassing the V_H -D- J_H segments. Strikingly, the V_H sequence is almost identical to that reported for the germ-line gene V (186.2)⁵, which encodes the V_H region of anti-NP antibodies in the C57BL/6 strain²³. Only two nucleotide differences were observed, one silent (within codon 81), and one producing an amino acid substitution (position 60). As all cross-hybridizing C57BL/6 V_H genes of the NP^b family have been isolated and characterized⁵, the unique copy of the reference V (186.2) gene must also be considered as a V_H germ-line gene of the anti-GAT antibodies in this strain (at least for those molecules that express the CGAT common idiotype specificity, and represent most anti-GAT antibodies). It should be stressed that, among the NP^b heavy chains, one was found to express the exact germ-line gene sequence, whereas others differed from the prototype sequence by several point mutations⁵. The anti-GAT V_H sequence that we describe is therefore closer to the NP^b germ-line gene than, presumably,

Fig. 1 Comparison of V_H sequences from F17.170.2 anti-GAT (CGAT) monoclonal heavy chain and the V (186.2) (NP^b) germ-line gene⁵. Numbering is according to ref. 26. Brackets indicate an ambiguous amino acid residue or base. A nucleotide in parentheses under the master sequence indicates a trace amount of this base.

Methods: The variable region sequence of the F17.170.2 anti-GAT monoclonal heavy chain was deduced by combined amino acid and nucleotide sequencing: the hybridoma was obtained by fusion of spleen cells from a (C57BL/6 $\times$ DBA/2) F₁ hybrid mouse with the NS1 myeloma cell line. Monoclonal antibodies were purified (from ascites) on a GAT-Sepharose column^{17,18}. The conditions used for automatic NH₂-terminal sequencing were as described elsewhere²⁷, except that, as the NH₂-terminus was blocked, the pyroglutamate amino peptidase (Sigma)²⁸. Hybridoma cells were transplanted into (C57BL/6 $\times$ DBA/2) F₁ mice and the resulting solid tumours were removed and stored at -70°C . The poly(A)⁺ RNAs were extracted directly from the frozen tumours by the LiCl-urea method²⁹, purified on an oligo (dT)-cellulose column, enriched for the H- or L-coding fraction by ultracentrifugation on a sucrose gradient (5–20%) and tested in the rabbit reticulocyte cell-free translation system. A synthetic oligonucleotide, dGGCCAGTGGATAGAC, complementary to a nucleotide sequence of the C_H1 domain close to the V-C junction³⁰ was used as a primer for the reverse transcriptase (obtained from Dr J. Beard). The nucleotide sequence was determined towards the 5' end of the mRNA, as far as codon 17, by using a modification of the dideoxy method²¹, with the mRNA as template²². As the amino acid sequence covered residues 1–36, there was extensive overlapping.



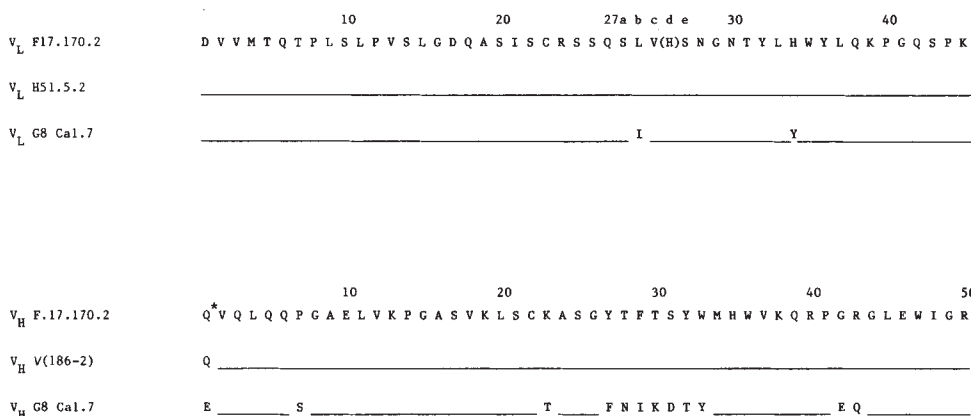
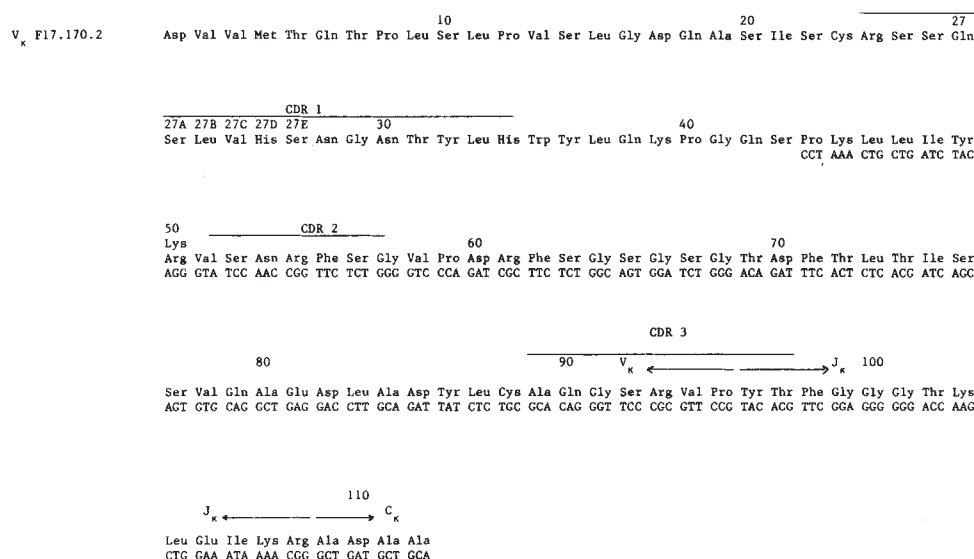


Fig. 2 Comparison of the first 50 residues of three V_H sequences (one NP^b, two CGAT) and three V_L sequences (all CGAT, as NP^b was of the λ type). The sequence of V (186.2) is taken from ref. 5; those of V_H and V_L G8 Cal.7 (BALB/c) and V_L H51.5.2 (BALB/c $\times$ DBA/2) from ref. 24. Q*, pyroglutamic acid.

Fig. 3 Combined amino acid and nucleotide sequences of the variable region of the F17.170.2 anti-GAT (CGAT) monoclonal light chain. The NH₂-terminal sequence was determined as far as residue 45. Cleavage of the light chain at the tryptophan residues³¹ allowed isolation of fragment 36–148, which was sequenced directly up to residue 62. Nucleotide sequence was performed as described in Fig. 1 legend, using a mRNA-enriched fraction coding for the light chain, and the oligomer dGTAGAAGGGTGGTAGGT, complementary to the beginning of the C_κ domain, as a primer. The nucleotide sequence was read up to codon 44. In position 50, the amino acid sequence indicates a lysine residue, and the nucleotide sequence an arginine residue.



most anti-NP V_H regions of antibodies expressing the NP^b idiotype. Figure 2 compares the first 50 amino acid residues of various heavy chains. Note that the sequence of the B6 anti-GAT/NP^b V_H region differs considerably from the homologous section of the prototype BALB/c anti-GAT heavy chain. Clearly, these two sequences must be encoded by different germ-line genes. The D region expressed by the B1.8 anti-NP antibody⁵ differed from that of the anti-GAT, but both anti-NP and anti-GAT antibodies used the same J_H2 gene.

Figure 3 gives the primary structure of the anti-GAT V -region κ -chain, also deduced from a combination of amino acid and nucleotide sequencing (positions 1–62 and 44–112, respectively). It differs completely from the anti-NP antibody light chains, which are of the λ type²³, but is identical, at least for the first 50 amino acid residues, to one of the two prototype sequences reported²⁴ for the GAT system in BALB/c or BALB/c $\times$ DBA/2 light chains. One discrepancy was found between the nucleotide and amino acid sequences at position 50, possibly due to enzymatic error during reverse transcription. As the hybridoma was derived from a (C57BL/6 $\times$ DBA/2) F₁ mouse, there is no way of knowing whether the light chains were of B6 or D2 origin, whereas the heavy chains expressed an allotypic marker of the B6 strain.

We recently reported that similar V_H gene products, presumably encoded by the same germ-line gene series, could yield anti-GAT antibodies of different fine specificity, depending on their association with V_κ domains encoded by very different genes²⁴. When associated with a V_κ 'CGAT' gene product, this V_H led to the production of an anti-GAT antibody recognizing primarily the GT (Glu-Tyr) determinants, and expressing the CGAT common idiotype specificity. When associated with a

V_κ 'GA-1' chain, antibodies directed against the GA (Glu-Ala) determinants were synthesized, and expressed another set of common idiotypic determinants, termed GA-1 (ref. 24).

The combinatorial association may now be extended to two unrelated antibody specificities, since the anti-NP and anti-GAT antibodies will use the same V_H gene product, associated with a λ or a κ light chain and will express the NP^b or the CGAT idiotype, respectively (Table 1). Obviously, the D region will contribute an additional element to diversity^{4,25} and participate in the antibody binding site itself. The basic point of our observation remains, however, that the same set of V germ-line genes (even though its diversity may be amplified by somatic events) may generate discrete antibody specificities through various (V_H -D- J_H) $\times$ V_L associations, thus decreasing the number of V genes needed for an efficient immunological repertoire.

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Early pre-B cells from normal and X-linked agammaglobulinaemia produce C_μ without an attached V_H region

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Differentiation of B lymphocytes follows an ordered pathway marked by somatic rearrangements of immunoglobulin heavy- and light-chain genes in conjunction with cellular transitions^{1–4}. Although low level constitutive transcription of the μ heavy-chain constant region (C_μ) genes may occur in early precursor cells⁵, activation of synthesis and translation of complete μ RNA is thought to accompany somatic rearrangements of DNA⁶. Cytoplasmic μ -chain protein serves as a marker for pre-B cells, the earliest cells committed to differentiation into B lymphocytes^{4,7,8}. μ -chain gene expression in pre-B cells precedes rearrangement and expression of light-chain genes^{5,8}. We now report that early human pre-B cells, Epstein-Barr virus transformed pre-B cells, and pre-B cell hybrid analogues, produce C_μ without the normally associated heavy-chain variable (V_H) region. Approximately 5% of normal pre-B cells from adult human bone marrow produce these incomplete μ -chains. Pre-B cells from three patients with X-linked agammaglobulinaemia are exclusively of this immature form, producing C_μ without associated V_H . This immune deficiency disease represents a block in differentiation secondary to failure to express V_H genes.

The immature pre-B-cell phenotype was identified by immunofluorescence of bone marrow pre-B cells with antiserum specific for the V_H protein. Preparation of this antiserum relied on the conserved framework of V_H regions common to all heavy-chain isotypes (see Fig. 1 legend). The reactivity of the anti- V_H was shown on a panel of well characterized B-cell lines, including a B-cell line which produced δ heavy chains but not light chains (refs 9–11, and J.S., unpublished results). All the B-cell lines were stained by anti- V_H . A T-cell line was not reactive. Preincubation of anti- V_H with Fv fragment (composed of V_H and V_L) isolated from pooled human IgG by the method of Lin and Putnam¹² completely eliminated fluorescent staining of immunoglobulin producing cells with anti- V_H , demonstrating the specificity of this antiserum for V_H .

Bone marrow from three patients with X-linked agammaglobulinaemia (XLA) was examined for presence of C_μ and V_H antigens. These patients have pre-B cells but no surface immunoglobulin (sIg) bearing cells (ref. 13, and J.S., unpublished results). In XLA bone marrow 12–28% of mononuclear cells were identified as pre-B cells. No cells containing V_H were found (Fig. 1d, e, f). Bone marrow from three normal individuals was also examined. To distinguish pre-B cells from mature B lymphocytes, cells were stained with peroxidase-conjugated antiserum for surface immunoglobulins, and then with rhodamine-conjugated antiserum for C_μ , and fluorescein conjugated anti- V_H (see legend to Fig. 1). Of the μ -positive cells 18–35% were cytoplasmic μ^+ and surface μ^- , that is, pre-B cells. 4–6% of these pre-B cells reacted with anti- μ but not with anti- V_H (Fig. 1a, b, c). These results are summarized in Table 1.

As the numbers of pre-B cells present in XLA bone marrow were small, Epstein-Barr virus (EBV) transformed cultures from XLA bone marrow were examined before being established as stable cell lines. 12–63% of cells from these cultures expressed C_μ . Of more than 500 μ^+ cells examined from each of these cultures, none reacted with anti- V_H . An attempt to expand pre-B cells from normal bone marrow by EBV transformation was unsuccessful because of overgrowth by B lymphocytes.

To study the molecular basis for the failure of the XLA and a small fraction of normal pre-B cells to express V_H antigen, bone marrow cells from XLA patients or fetal liver cells from 17-week abortuses were fused with the LSM 2.7 human myeloma cell line¹⁴. This myeloma cell line does not produce immunoglobulin (J.S., unpublished results). 10^7 LSM 2.7 myeloma cells were fused with 10^8 bone marrow or fetal liver cells with 50% polyethylene glycol and hybrid cells isolated in hypoxanthine-aminopterin-thymidine half-selective medium as described by Littlefield¹⁵, modified by Davidson¹⁶. Hybrid colonies from XLA bone marrow were uniformly of the $C_\mu^+V_H^-$ phenotype. Several normal fetal liver hybrid clones with the $C_\mu^+V_H^-$ phenotype were identified and one hybrid (LSH6) was selected for molecular characterization.

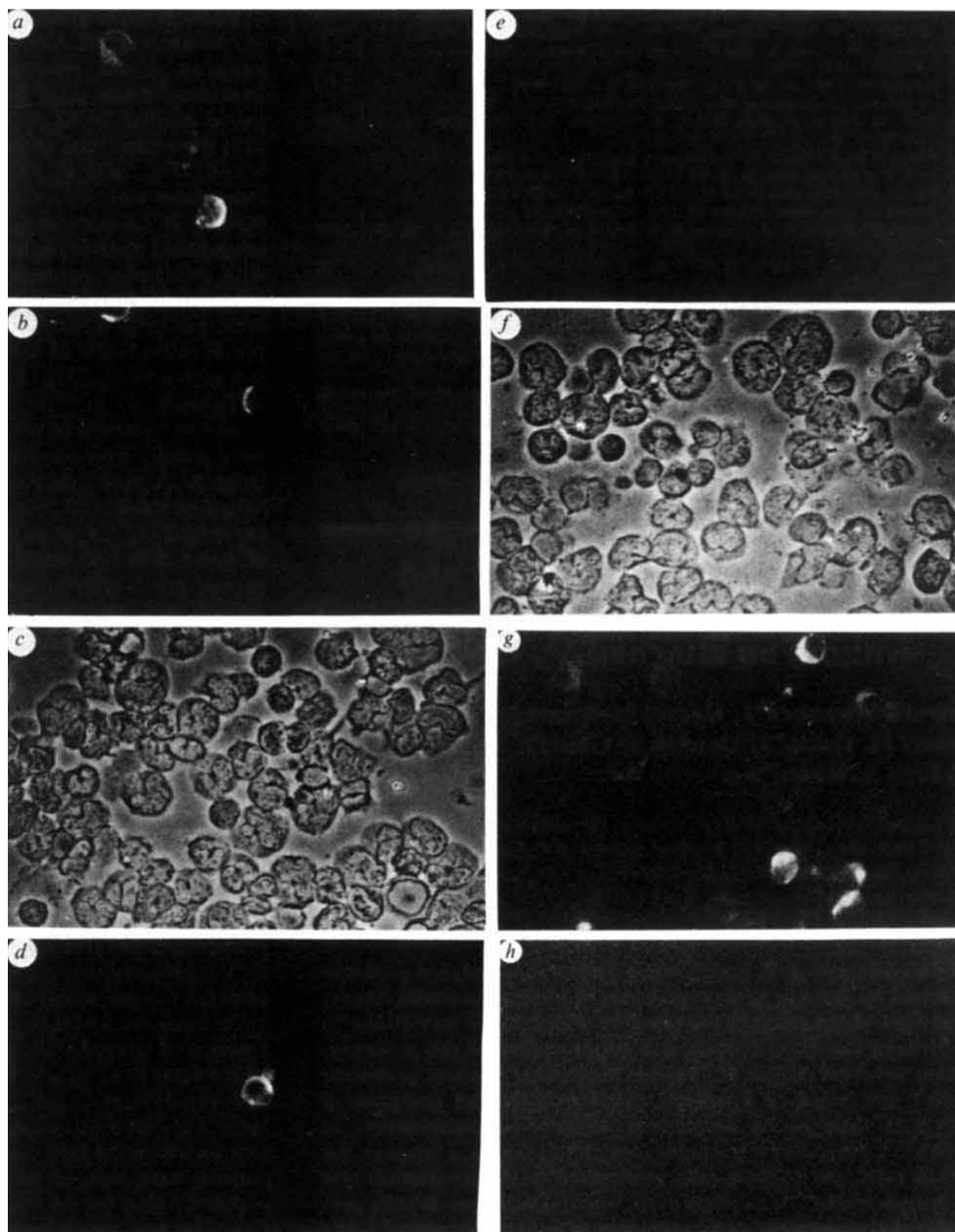
To examine whether the absence of V_H antigen was due to absence of polypeptide sequence, we isolated RNA from the LSH6 fetal liver hybrid for translation in a rabbit reticulocyte cell-free system¹⁷. μ -Chain protein immunologically precipitated and electrophoresed in SDS-acrylamide gels migrated as two bands at 54,000 and 57,000 molecular weight, in comparison with one of 66,000 molecular weight for complete μ -chain coded for by the normal B-cell line RPMI 1788 (Fig. 2a). The two bands suggest secreted and membrane species of C_μ RNA. Translation of RNA from the XLA pre-B cell hybrid LSM-T gave a μ -chain of the same reduced size (data not shown). Electrophoresis of immunologically precipitated μ -chain isolated following internal labelling of the LSH6 cells⁹ also migrated as a band of 56,000 molecular weight (Fig. 2b).

The basis for production of foreshortened μ -chain polypeptide was examined in mRNA from three XLA pre-B cell hybrids and the LSH6 normal fetal liver hybrid. In Northern blot analysis¹⁸ with the pmu HM2 cDNA clone, the μ -chain RNA migrated at 2.0–2.1 kilobases (kb), compared with the 2.3–2.4 kb μ RNA from a normal B-cell line (Fig. 3a)¹⁹. This size reduction corresponds to the 300 nucleotide size of the V_H region²⁰.

The reduction in size of the pre-B cell μ RNA was localized by primer-extension analysis²¹ to the 5' portion (V_H region) of the mRNA. We prepared a 61 nucleotide primer from the $CH1$ region of the pmuHM2 μ cDNA clone (see legend to Fig. 4). The primer was composed of nucleotides 47–108 of the μ $CH1$ domain. Following hybridization with cellular RNA²², reverse transcriptase (avian myeloblastosis) was used to copy the RNA to its 5' terminus²³. Where μ mRNA from a normal B-cell line (SM14) gave primed cDNA fragments 550 nucleotides long, μ mRNA from the LSH6 fetal liver hybrid and the three XLA

Fig. 1 Pre-B-cell immunofluorescence. Bone marrow cells from normal individuals were stained with peroxidase-conjugated anti-serum to human immunoglobulin for surface immunoglobulin, placed on microscope slides with a cytocentrifuge, fixed at -20°C for 15 min with acetone, washed extensively in phosphate-buffered saline without divalent cations (PD) for 30 min, and stained with fluorescein isothiocyanate (FITC, Baltimore Biological Labs) conjugated anti- V_H and tetramethylrhodamine isothiocyanate (TMRITC) conjugated anti- μ^{28} .

After staining, the cells were reacted with *o*-phenylenediamine in 0.1 M citrate buffer, pH 4.5, to develop the peroxidase-conjugated antibody to sIg and so to distinguish B lymphocytes from pre-B cells. *a*, Normal bone marrow with TMRITC (anti- μ) fluorescence; *b*, normal bone marrow with FITC (anti- V_H) fluorescence: only one of the two anti- μ cells expressed V_H region; *c*, phase contrast photomicrograph of the field. Bone marrow cells from a patient with XLA were placed on microscope slides with a cytocentrifuge, fixed in acetone, and stained with FITC anti- V_H and TMRITC anti- μ ; *d*, XLA bone marrow with TMRITC (anti- μ) fluorescence; *e*, XLA bone marrow with FITC (anti- V_H) fluorescence; *f*, phase contrast photomicrograph of the field. Clone 4.4 cells from LAZ 007, a normal B-cell line, were stained with FITC anti- V_H alone (*g*), or after preincubation of the antiserum with Fv protein¹² in fourfold molar excess (*h*). Antiserum specific for V_H was prepared from the IgG fraction of goat antiserum to the Fab' fragment of human IgG (Cappel Labs). The IgG was pepsin digested, chromatographed in Sephadex G-150 to isolate the Fab₂' fragment, affinity purified on human IgM coupled to Sepharose 4B, and then conjugated with FITC (Baltimore Biological Labs)²⁸. The critical step in preparation of anti- V_H is isolation of the antibodies which react with both IgG Fab' and IgM. Specificity of the anti- V_H was demonstrated by elimination of reactivity by prior absorption with Fv fragment. Fv fragment was prepared by the cold pepsin digestion method of Lin and Putnam¹⁴. Briefly, pooled human IgG was treated with pepsin (1 mg per 25 mg) for 48 h at 4°C with addition of more pepsin after 24 h, followed by chromatography in Sephacryl S-300. Two peaks corresponding to Fab' and Fv by molecular weight, were isolated. A second run of the Fv peak through Sephacryl confirmed the migration pattern.



pre-B-cell hybrids gave primed cDNA fragments 240 nucleotides long (Fig. 4). These results demonstrate that the reduction in size of the mRNA is due to absence of the V_H region.

To ensure that the reduction in size of the pre-B cell μ RNA did not result from deletion of a portion of the C_μ gene, we performed restriction map analysis of cellular DNA. Southern blot hybridization of the DNA²⁴ with μ -chain cDNA probe showed that the C_μ genes were of normal size (Fig. 3b).

Some pre-B cells from normal individuals, particularly pre-B cells from fetal liver, and all pre-B cells from three patients with XLA, produced incomplete μ heavy chains. Five per cent of normal pre-B cells had a $C_\mu^+V_H^-$ phenotype, associated with a larger percentage of cells in fetal liver. All of the pre-B cells from XLA three patients had the $C_\mu^+V_H^-$ phenotype. A human-human fetal liver-pre-B-cell hybrid analogue and human-human pre-B-cell hybrids from the patients with XLA

expressed C_μ without associated V_H . The μ -chain mRNA produced by these hybrids were 200–300 bases smaller than the normal μ mRNA produced by a B-cell line. By primer-extension analysis, this reduction in size was found to represent 300 bases at the 5' terminus of the molecule. Translation of this RNA, both *in vitro* and *in vivo*, yielded μ -chains which were reduced in size compared with μ -chain produced by normal B-cell lines. This reduction in size, both of RNA and of protein, was sufficient to include the entire V_H region. Southern blot analysis indicated that the C_μ gene in these cells is intact.

We propose that pre-B cells producing μ -chain without V_H region represent the earliest stage of identifiable B-lymphocyte precursor. Production of this incomplete molecule may precede translocation of a particular V_H region gene 5' to the $D-J_H-C_\mu$ gene locus. We have not determined whether D and J_H have undergone rearrangement from the embryonic configuration in

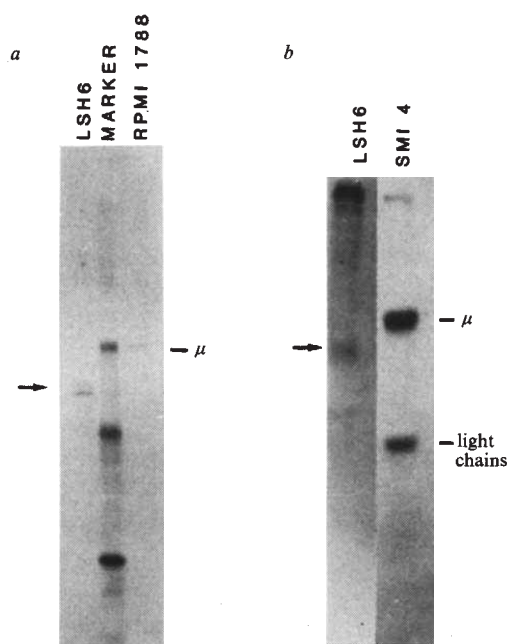


Fig. 2 *a*, Translation of C_μ RNA. Total cell RNA from the LSH6 fetal liver hybrid was translated in a rabbit reticulocyte system (Amersham) in the presence of ^{35}S -methionine. The resulting proteins were incubated with antiserum to IgM (Dako), followed by *Staphylococcus aureus* containing A protein. The precipitated proteins were redissolved in 0.1% SDS, 0.1% 2-mercaptoethanol, and electrophoresed in a 10% SDS-acrylamide gel according to Laemmli²⁹. ^{14}C -labelled bovine serum albumin, ovalbumin and carbonic anhydrase (66,000, 46,000 and 29,000 molecular weight respectively) were electrophoresed in an adjacent lane. RNA from RPMI 1788, a normal B-cell line, was translated, immunoprecipitated, and electrophoresed in parallel to yield full sized μ -chains. *b*, *In vivo* production of μ -chains. LSH6 fetal liver hybrid and SMI 4 normal B-cell line were incubated with ^3H -labelled leucine in tissue culture medium with half the usual amount of leucine for 4 h (refs 9, 11). The cells were lysed by addition of NP40 to 0.5%, the nuclei were removed by centrifugation, and the immunoglobulins from the resulting cell lysate were isolated by precipitation with antiserum to IgM followed by *S. aureus*. The precipitates were redissolved in 0.1% SDS, 0.1% 2-mercaptoethanol, and electrophoresed in a 7.5% acrylamide gel with SDS-phosphate buffer³⁰.

these pre-B-cell hybrids. However, preliminary results with two pre-B-cell lines with this phenotype from a single XLA patient suggest that their J_H region genes have undergone rearrangement from the embryonic configuration.

The immunoglobulin molecule produced by these early pre-B cells is composed either of C_μ alone, or of C_μ in association with rearranged D and J_H region genes, corresponding to the third hypervariable region of V_H (ref. 22). A sequence which could serve as promoter for this incompletely rearranged gene has not been identified, although Kemp *et al.*⁵ have shown that the C_μ gene is transcribed before rearrangement in related cell types. The C_μ RNA transcripts described by Kemp *et al.*⁵ are not translated^{25,26}. Physiologically, a D - J_H - C_μ molecule may represent a primordial antibody molecule, which would have lowered antibody affinity in conjunction with broadened specificity. This primordial antibody molecule might serve as an early antigen receptor, providing a basis for selecting particular clones for expansion. Alternatively, translation of C_μ completely lacking the V_H region protein may serve as a regulatory molecule controlling rearrangement of a V_H gene. Determination of the function, if any, of these shortened μ -chain products requires further study.

Table 1 Expression of V_H region protein by pre-B cells

		% Cytoplasmic μ^+ surface μ^- cells*	% V_H^+ cells
Normal	1	18	94.0
	2	36	95.1
	3	31	94.4
XLA	N	12	0
	A	28	0
	T	24	0

Bone marrow slides were stained with anti- μ and anti- V_H . From normal individuals, surface immunoglobulin was stained with peroxidase. More than 300 pre-B cells were examined for V_H region protein from each individual. N, A and T are the initials of the last names of the three XLA patients.

* Per cent of mononuclear cells.

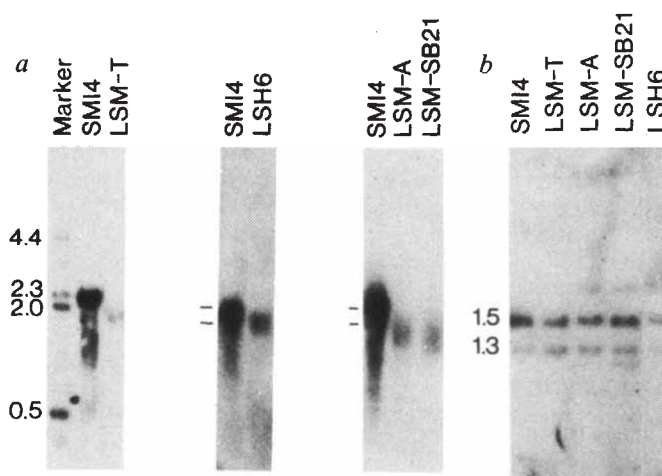


Fig. 3 *a*, Northern blot hybridization of RNA from normal and XLA pre-B-cell hybrids. RNA was isolated from 5×10^8 cells of each hybrid by lysis in guanidine-HCl, followed by differential precipitation in ethanol, and phenol: (chloroform/isoamyl alcohol) [1: (24:1)] extraction³¹. 20 μg of total RNA was treated with glyoxal and electrophoresed in 1.2% agarose gels as described by Thomas¹⁸. RNA was transferred from the gel to nitrocellulose in $20 \times \text{SSC}$, pH 7.5, air dried and baked at 80°C for 2 h. Clone pmuHM2, representing cDNA coding for C_μ (1.6 kb starting at base 30 of the $CH1$ domain) cloned in PAT 153 (H.M., unpublished results) was used as probe. Total plasmid was digested with *Pst*I and labelled with ^{32}P -dCTP using T4 DNA polymerase³². Filters were hybridized in 50% formamide, $1 \times \text{Denhardt's}$ ³³, 100 μg sonicated salmon sperm DNA, 0.1% SDS with $5 \times \text{SSC}$, and washed to $0.1 \times \text{SSC}$ with 0.1% SDS at 60°C . Filters were autoradiographed for 24–72 h at -70°C with intensifying screen. SMI4 is a normal B-cell line which produces IgM. LSH6 is a human hybrid formed with normal fetal liver. LSM-T, and LSM-A are human hybrids formed with bone marrow from XLA patients A and T. LSM-SB21 is a human hybrid formed with the pre-B-cell line SB21 derived from bone marrow of XLA patient N by EBV transformation. λ DNA digested with *Hind*III was used as molecular weight marker on each gel. *b*, Southern blot of genomic DNA from normal and XLA pre-B-cell hybrids. Genomic DNA was isolated from 2×10^8 cells as previously described³⁴. 15 μg of *Eco*RI digested DNA was electrophoresed per lane in 1.0% agarose. DNA was transferred to nitrocellulose by the method of Southern²⁴, and hybridized to the nick-translated C_μ cDNA insert of clone pmuHM2 in $6 \times \text{SSC}$ at 68°C for 24 h before stringent washing in $0.1 \times \text{SSC}$ at 68°C . Cell lines are as described in *a*.

In this model of pre-B-cell differentiation, our results suggest that XLA occurs secondary to a failure to differentiate, associated with a failure to translocate V_H -region genes. Synthesis of the complete μ -chain including the V_H region may be

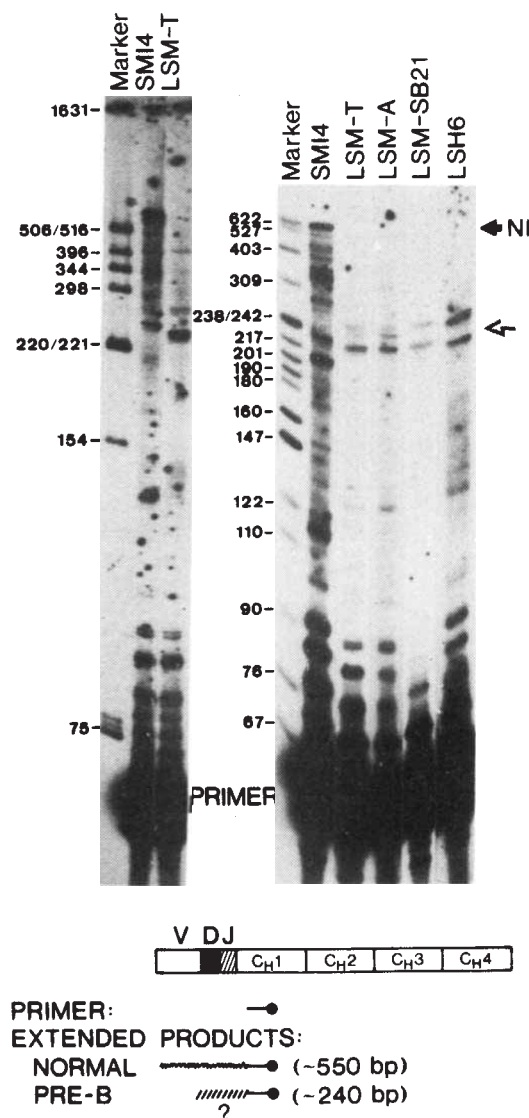


Fig. 4 Primer-extension of C_{μ} $CH1$ fragment on pre-B cell RNA. RNA was isolated from normal and XLA pre-B cell hybrids as described in the legend to Fig. 2. C_{μ} cDNA insert from clone pmuHM2 was digested with *HinfI*, 5'-end-labelled with ATP, using polynucleotide kinase³⁵, and digested with *EcoRI*. A 61 nucleotide fragment corresponding to bases 47–108 of the $CH1$ domain of μ -chain, was isolated from a 10% acrylamide gel. Labelled primer and 25–50 μ g of total cell RNA were denatured in hybridization buffer²³ and incubated for 20 h at 51 °C followed by ethanol precipitation. Hybridized primer was extended with AMV reverse transcriptase according to Treisman *et al.*²⁵. Reactions were phenol extracted, ethanol precipitated and electrophoresed for 2 h at 2,000 V in 8% acrylamide gels containing 6 M urea³⁶. Size markers were plasmid pBR322 digested with either *HinfI* or *MspI* and 5'-end-labelled. Full-length, normal B-cell line product is marked by a closed arrow. Shortened pre-B-cell line products are marked by an open arrow. SMI 4 is a normal IgM-producing B-cell line, LSH6 is a normal fetal liver pre-B-cell hybrid, LSM-T, LSM-A and LSM-SB21 are XLA pre-B-cell hybrids.

required before expression of light-chain genes, or before switching from one heavy-chain isotype to another. Alt *et al.*⁶ proposed a similar regulatory role for immunoglobulin light chains in heavy-chain isotype expression. The X-linkage of this disease suggests that the failure to translocate V_H region genes is not due to an abnormality of the immunoglobulin heavy-chain structural genes which are on chromosome 14 (ref. 27). Rather, enzymes specific for translocation of V_H genes or regulatory

sites necessary for pre-B cells to differentiate to a stage utilizing these enzymes may be encoded on the human X chromosome.

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Detection of a novel human class II HLA antigen

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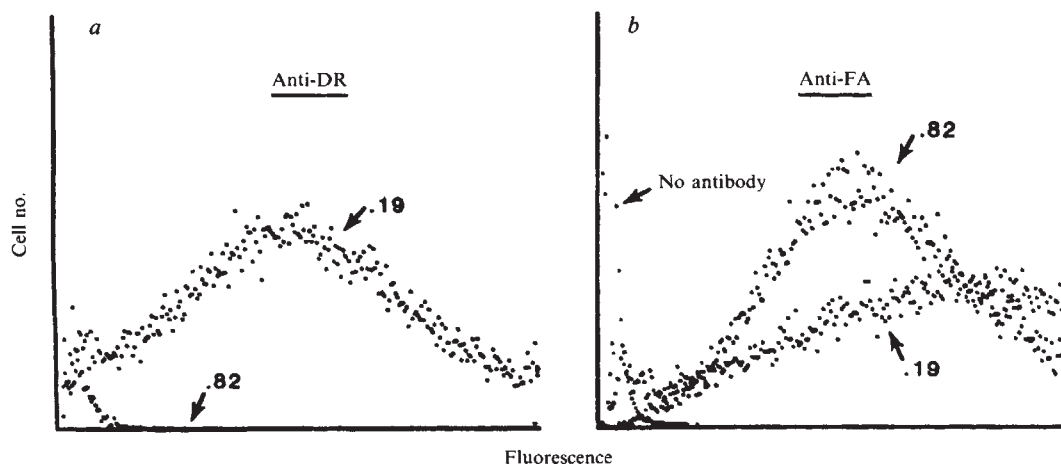
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Genetic, molecular and cellular analyses of the *HLA-D* region of the major histocompatibility complex (MHC) in man have led to the definition of three different products. Two of these, DR and MB (the latter also known as DC (ref. 1) and LB-E (ref. 2)) are defined with serological reagents; the third, known as SB (ref. 3) and PL-3 (ref. 4) is defined with primed lymphocyte typing (PLT) cells⁵. The classical features attributed to *HLA-D* region encoded (class II) molecules are that they are cell-surface dimers consisting of a structurally conserved α -chain noncovalently associated with a polymorphic β -chain and that they are found primarily on B lymphocytes, some monocyte populations, endothelial and certain other cells⁶. Using these criteria a monoclonal antibody, B7/21, was described as reactive with HLA-DR (ref. 7). We have now re-evaluated B7/21 antibody reactivity using mutant lymphoblastoid cell lines. It appears that this antibody does not react with the molecularly defined D region products described to date but instead, recognizes a class II antigen with distinctive molecular characteristics. We provisionally refer to this antigen as FA.

Fig. 1 Anti-FA antibody binds to DR and MB null lymphoblastoid cell line mutants. FACS analysis of the binding of anti-DR antibody (L243) (a) and the anti-FA antibody (B7/21) (b) to mutants derived from human lymphoblastoid cell line, LCL 721. LCL 721 has the haplotypes HLA-[A1, -B8, -MB2, -DR3, -SB4]; GLO2 and HLA-[A2, -B5, -MB1, -DR1, -SB2]; GLO1. Mutant .19 has a γ -ray-induced deletion of the haplotype that includes HLA-[A1, -B8, -MB2, -DR3, -SB4] but still expresses the haplotype that includes HLA-[A2, -B5, -MB1, -DR1, -SB2]⁸. Mutant .82 was derived from mutant 45 (a mutant similar to .19) by γ -irradiation and selection with complement and the anti-DR monoclonal antibody, L243. Mutant .82 does not bind monoclonal antibodies that are known to recognize DR and MB antigens, that is, it is DR null and MB null¹¹. Additional confirmation of DR and MB determinant loss on .82 and similar mutants was obtained using recognized serological tissue typing reagents at both the University of Minnesota Hospital Tissue Typing laboratories and the Blood Bank of Southern Wisconsin, Inc.¹⁵. Fluorescence intensity is represented on a linear scale in a (gain 1.5) and b (gain 4). Control experiments demonstrate that the fluorescein-conjugated goat anti-mouse IgG shows minimal binding to the LCL mutants both in the absence of antibody (b) and in the presence of a non-binding monoclonal antibody or control serum (data not shown). In these experiments cells were washed three times with buffer (phosphate-buffered saline (PBS), 2% fetal calf serum, 0.02% azide) and incubated at 4°C with excess antibody. After 30 min the cells were washed and reincubated with either pretitrated and airfuge-clared (20 min at 4°C, Beckman) fluorescein-conjugated goat anti-mouse IgG antibodies (Meloy) or fluorescein-conjugated Fab fragments of rabbit anti-mouse IgG (Cappel). Following removal of the fluorescein-conjugated second antibody, 40,000 cells were analysed in a Becton-Dickinson FACS IV.



In these studies FA antigen expression was investigated using both the anti-FA monoclonal antibody, B7/21 (ref. 7) and mutants of the lymphoblastoid cell line, LCL 721 (carrying haplotypes HLA-[A1, -B8, -DR3, -MB2, -SB4], GLO2 and HLA-[A2, -B5, -MB1, -DR1, -SB2]; GLO1 (refs 8–11). These mutants have lost expression of various markers associated with one or both HLA haplotypes and were derived following γ -irradiation and immunoselection with cytotoxic alloantisera and monoclonal antibodies. Such mutants have been used to map determinants encoded by *HLA* genes and recognized by primed lymphocyte typing reagents, including the mapping of the *SB* locus in the MHC between *HLA-DR* and glyoxylase (*GLO*)^{10,12,13}. Also these mutants offer great potential for mapping determinant(s) recognized by monoclonal antibodies (for example, see refs 11, 14). We have used two categories of mutants: (1) those that have lost one complete HLA haplotype from *HLA-A* to *GLO*, inclusive, but retain the second haplotype and, (2) those that are DR null or DR and MB null (that is, do not express detectable DR or MB products), having been derived from a category I mutant following a second mutagenic exposure to γ rays and immunoselection against the remaining DR specificity^{11,15}. The following evidence suggests that the anti-FA monoclonal antibody does not react with DR or MB products.

We used a fluorescence-activated cell sorter (FACS IV, Becton-Dickinson) to study the binding of the FA antibody to mutants of LCL 721. The FACS analyses presented in Fig. 1 use the DR and MB positive mutant .19 and, for comparison, the DR and MB null mutant .82. This latter mutant is DR and MB null according to typing with monoclonal antibodies including the MB-1 antibody, Genox 3.53 (ref. 11), and a total of 17 DR and 13 MB allospecific serological reagents¹⁵. Furthermore, Southern blotting analyses of DNA from mutant .82 with an MB α -chain probe shows that there is a homozygous deletion of, at least, the MB α -chain gene¹⁶. Figure 1a demonstrates that DR antibody, L243, which recognizes a monomorphic DR determinant^{17–19}, shows no binding over background to mutant .82, as expected, but does bind to mutant .19. In contrast, the FA antibody binds to both mutants .82 and .19 (panel b). Similar binding results were obtained with five out of the five DR null mutants tested, three of which were also MB null.

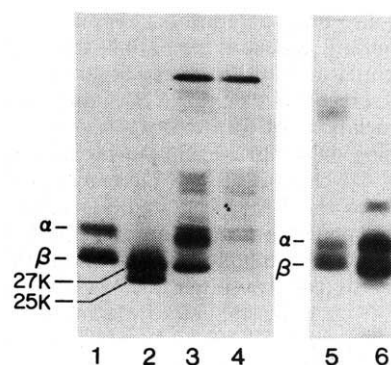


Fig. 2 The molecular structure of FA differs from that of DR and MB. SDS-PAGE analysis of molecules immunoprecipitated from NP40 lysates of ¹²⁵I-lactoperoxidase labelled LCL 721 parental (non-mutant) cells by the monoclonal DR antibody L243 (lane 1), anti-FA antibody, B7/21 (lane 2), an anti-MB-1 monoclonal antibody, Genox 3.53 (lane 3)²² (a gift from Dr J. Strominger), a control immunoprecipitate for Genox 3.53 antibody using a non-immune serum (lane 4), rabbit anti-p23,30 serum (a gift from Dr J. F. Kaufman) (lane 5) and the monoclonal antibody TDR31.1²¹ (provided by Dr M. Crumpton) (lane 6). The presence of nonspecific bands in Genox 3.53 immunoprecipitates, observed in lane 3, has been reported by others¹⁸; however, in comparison with the control immunoprecipitate in lane 4, the α - and β -MB chains are clearly distinguishable, migrating slightly ahead of the DR α - and β -chains (lane 1), consistent with other reports¹⁸. In these experiments washed cells were surface labelled with ¹²⁵I using the lactoperoxidase procedure described by Ledbetter *et al.*²⁷. ¹²⁵I-labelled cells were then solubilized in 0.5% NP40, pre-cleared with *Staphylococcus aureus* and immunoprecipitated as described previously²⁸, with the exception that immunoprecipitation with Genox 3.53 antibody was conducted as described by Shackelford *et al.*¹⁸. Washed immunoprecipitates were solubilized using Laemmli sample buffer²⁹ and separated on 10% SDS-polyacrylamide slab gels also as described previously²⁸. Following electrophoresis gels were fixed, dried and autoradiographed at -70°C using Cronex 2 (Dupont) intensifying screens and Kodak XAR-5 film.

The FA antigen structure is also distinguishable from DR and MB locus products. SDS-polyacrylamide electrophoresis (PAGE) analysis of molecules immunoprecipitated by the FA antibody from ^{125}I -lactoperoxidase cell-surface labelled LCL 721 parental (non-mutant) cells reveals two closely migrating chains with molecular weights of approximately 25,000 (25K) and 27,000 (27K) (Fig. 2, lane 2). This is in contrast to the α - β -chain dimer characteristic of class II antigens immunoprecipitated by the DR antibody, L243 (lane 1). The reactivity of L243 antibody has been extensively investigated and, in most cases, includes all recognized subsets of mature cell-surface DR β -chains¹⁸⁻²⁰. Bands similar or identical to those in lane 1 are also immunoprecipitated from LCL 721 by a rabbit anti-p23,30 serum which is also broadly reactive with known DR subsets¹⁹ (lane 5). Furthermore, another DR antibody, TDR31.1, which is reported to immunoprecipitate two β -chains²¹, immunoprecipitates an α - and β -chain complex from the ^{125}I -labelled LCL 721 (lane 6) similar to that precipitated by L243 antibody (lane 1). Figure 2, lane 3, shows that the FA structure is also distinct from the α - β -chain complex of the MB-1 product immunoprecipitated by Genox 3.53 antibody^{18,22}. Reciprocal immunodepletion experiments lend further support to this separation of FA from DR and MB products by demonstrating that FA and L243 determinants are located on mutually exclusive populations of molecules (data not shown). Additionally, immunodepletion of LCL 721 lysates of FA does not deplete the lysates of anti-p23,30 or Genox 3.53 reactive material (data not shown).

The apparent absence of an α -chain structure in FA immunoprecipitates from lactoperoxidase ^{125}I -radiolabelled LCL 721 has also been noted in FA immunoprecipitates from 18 other similarly labelled lymphoblastoid cell lines. Two possibilities could account for these observations: (1) the FA antibody may selectively bind to FA β -chains in a manner which induces dissociation of pre-existing α - β -chain complexes thereby, causing only FA β -chains to be immunoprecipitated or, (2) a cryptic α -chain is present but is undetected because it is inaccessible to the ^{125}I -lactoperoxidase radiolabelling procedure. To distinguish between these two, FA was immunoprecipitated from metabolically (^{35}S -methionine) labelled LCL 721 and also from two other lines: NOB, a DR2 homozygous cell line from a consanguineous marriage which expresses a single FA chain of 25K and LL4, a DR4 homozygous LCL on which only the 27K FA chain is detected. The data presented in Fig. 3a demonstrate that when FA is immunoprecipitated from ^{35}S -methionine-labelled LCL 721, in addition to the usual 25K and 27K bands, a strongly labelled α -chain is detected (lane 2) which migrates slightly ahead of the corresponding DR α -chain (lane 3). Similarly, an anti-FA precipitable α -chain was not detected in ^{125}I -lactoperoxidase-labelled NOB and LL4 cell lysates but ^{35}S -methionine incorporation reveals the presence of a distinct α -chain structure (compare lanes 4, 5 and 8, 9). In the original description of B7/21 (anti-FA) antibody reactivity (and in subsequent experiments), a weakly labelled α -chain-like structure was immunoprecipitated from the ^{125}I -lactoperoxidase-labelled lymphoblastoid cell line, Jijoye⁷. Studies by us (A.J.W., F.H.B.) of more than 18 other similarly labelled lymphoblastoid cell lines have, however, failed to detect an equivalent band and, thus, this difference apparently reflects an unusual aspect of the Jijoye cell line.

Comparative isoelectric focusing (IEF) analyses of ^{35}S -methionine-labelled FA and DR complexes isolated from LCL 721, NOB and LL4 show that the FA α -chain has a more acidic equilibrium focusing position than the corresponding DR α -chains (Fig. 3b). Furthermore, the FA β -chains did not migrate or resolve adequately in this and other experiments (Fig. 3b, triangles) using various IEF conditions. We thus suspect that FA β -chains are either markedly less soluble, or are perhaps intrinsically more prone to aggregate, than are DR β -chains (Fig. 3b) and MB β -chains (data not shown).

Chromosomal mapping of the locus of loci for FA β -chains was obtained from molecular studies of FA expression on

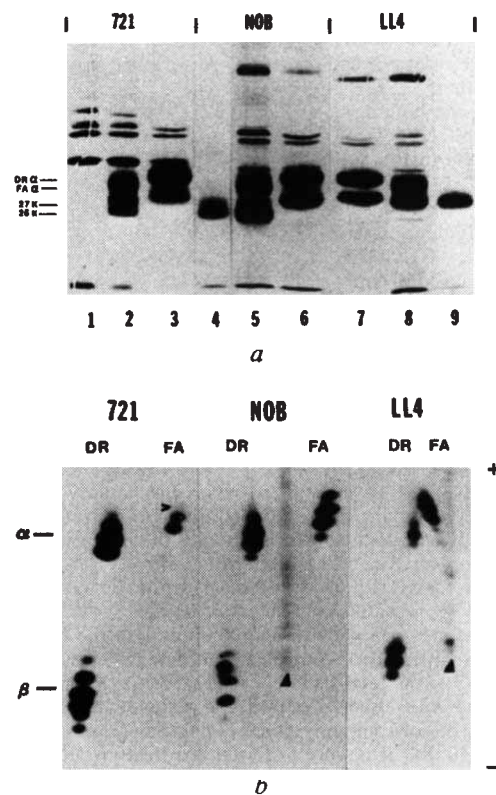


Fig. 3 Detection and characterization of FA α -chain. SDS-PAGE analysis of FA and DR antigens immunoprecipitated from LCLs 721, NOB and LL4 labelled with either ^{35}S -methionine or by the ^{125}I -lactoperoxidase procedure. Lanes 2, 5, 8 contain FA immunoprecipitated from detergent lysates of ^{35}S -methionine-labelled LCL 721, NOB and LL4, respectively. For comparison, FA immunoprecipitated from ^{125}I -lactoperoxidase labelled NOB and LL4 lysates is shown in lanes 4 and 9. Lanes 3, 6 and 7 contain DR antigens immunoprecipitated by L243 antibody from ^{35}S -methionine-labelled LCL 721, NOB and LL4 respectively. Lane 1 is a control immunoprecipitate done in the absence of antibody using ^{35}S -methionine-labelled LCL 721 cell lysate. Flat-bed isoelectric focusing (IEF) analysis of FA and DR α - and β -chains derived from one-dimensional SDS-PAGE separations of antigens immunoprecipitated from ^{35}S -methionine-labelled LCL 721, NOB and LL4s. Horizontal arrowhead denotes a minor FA band and bands above triangles originated from FA β -chains. Improved FA β -chain focusing was not obtained by variations in equilibrium conditions, urea concentrations, ampholine mixes, V h^{-1} combinations or anodic or cathodic sample application. In these experiments cells were labelled for 16 h with ^{35}S -methionine (Amersham; 2.0 mCi per 15×10^6 cells) in methionine-free RPMI 1640 containing 7.5% FCS. Radiolabelled cells were lysed, immunoprecipitated and SDS-PAGE gels prepared as described in Fig. 2 legend. Approximately 4×10^6 c.p.m. are required to obtain equal intensity of autoradiographic development between DR and FA lanes. For IEF, fluoresceinated and dansylated IgG and DNase marked the regions of the first-dimensional gel containing DR and FA chains to be excised. Sample equilibration and IEF was then conducted essentially as described by Shackelford *et al.*¹⁸ with the following modifications: equilibrated first-dimensional gel samples were cast directly into the IEF gel at the anode and sealed in place by polymerizing the gel onto the hydrophilic surface of a Gel-Bond PAG film (FMC Corporation). Focusing was conducted at 7°C using a Hoefer Isobox (Hoefer Corporation) at 20–30 W constant-power to a maximum of 1,800 V for 5 h. Total power applied was $\sim 8,500 \text{ V h}^{-1}$. Following electrophoresis, or IEF, all gels were fixed and enhanced with diphenyl-oxazole³⁰ before autoradiography with Kodak XAR-5 film at -70°C .

^{125}I -lactoperoxidase-labelled category I full-haplotype loss mutants of LCL 721. Full-haplotype loss mutants do not express the antigens encoded by one haplotype from, and including, HLA-A to the GLO locus^{8,9,11}. When FA molecules are immunoprecipitated from mutant .39, which no longer

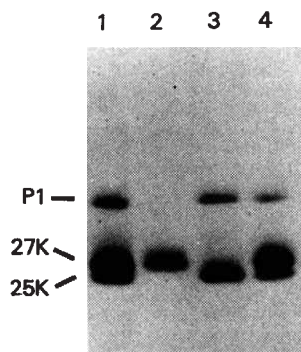


Fig. 4 The molecular structure of FA on full haplotype loss LCL mutants. SDS-PAGE analysis of antigens immunoprecipitated with the FA antibody from cells surface-labelled with ^{125}I . Lane 1, LCL 721 (parental, non-mutant); lane 2, mutant .39, which expresses only the HLA-[A2, -B5, -MB1, -DR1, SB2] haplotype; lane 3, mutant .120, which expresses only the HLA-[A1, -B8, -MB2, -DR3, -SB4] haplotype. Lane 4 contains pooled FA antibody immunoprecipitates from mutants .39 and .120. Band, P1 is suspected of being actin, a common contaminant in immunoprecipitates from human lymphoblastoid lines, as it co-migrates with class I antigens and is present in nearly all immunoprecipitates regardless of the antibody specificity. Methods were as described in Fig. 2 legend.

expresses the HLA [A1, -B8, -MB2, -DR3, -SB4] haplotype, the lower molecular weight, 25K, β -chain is not detected but the 27K β -chain is expressed (Fig. 4, lane 2). Conversely, full-haplotype loss mutants affecting the other haplotype, such as mutant .120, have lost the 27K β -chain and express only the 25K β -chain (lane 3). This observation has been confirmed with two other independently derived mutants similar to .39 and one other similar to .120. The two β -chains characteristic of the non-mutant, parental LCL are observed after pooling immunoprecipitates from mutant .39 and .120 (lane 4). These data strongly suggest that the locus for the FA β -chains maps to the short arm of chromosome 6, since karyotype analysis of full-haplotype loss mutants has revealed the presence of microscopically visible deletions in this region of chromosome 6 (refs 8, 9 and unpublished observations). These deletions include losses of HLA class I and class II DNA as confirmed by Southern blotting analysis of restriction enzyme DNA fragments (refs 16, 23, 24 and unpublished observations). Evidence for a more specific localization of an FA locus in the MHC was obtained from two mutants of LCL 721 which do not bind the FA antibody. These mutants have a homozygous deletion of the region between a SB gene and HLA-B, inclusive (R. DeMars *et al.*, manuscript in preparation). All the evidence is therefore consistent with the presence of, at least, a locus encoding FA β -chain within the MHC. In addition, because only one FA β -chain is lost in the full-haplotype loss mutants, and the identity of the β -chain retained is dependent on the haplotype deleted, the data in Fig. 4 also suggests that each FA β -chain on LCL 721 may be an allelic product of one locus.

The above data suggest that FA is distinct from known DR and MB products. The relationship of FA to SB^{3,10,14,25,26}, the third locus attributed to the HLA-D region, cannot be readily resolved at present. Limited SB N-terminal amino acid sequence data are available⁶ but SB product susceptibility to different radiolabelling procedures and its migration characteristics on IEF analysis have not been reported. Indeed, few comparative data are available concerning SB behaviour in these and other systems of analysis, perhaps due to the difficulties encountered in isolating SB using available serological reagents (A.J.W., unpublished observations).

To our knowledge, FA is the first human class II antigen described which displays an apparently cryptic, lactoperoxidase-inaccessible, α -chain as well as a distinct molecular weight polymorphism of the β -chain. We are currently pursuing

further studies on the structural relationship between FA and other HLA-D region products.

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Disruption of microfilament organization after injection of F-actin capping proteins into living tissue culture cells

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Capping proteins are F-actin binding proteins which interfere with the *in vitro* growth of an actin filament by blocking one of its ends (for recent reviews see refs 1-3). The majority of such proteins described so far 'cap' the fast-growing (positive) end of the polar filament, thus reducing the velocity of filament growth while increasing the number of filaments being formed *de novo* from a monomer pool⁴⁻¹⁰. We have studied the effects of capping proteins on the organization of actin filaments in living tissue culture cells by microinjection in conjunction with fluorescence, reflection contrast and electron microscopy. Our results, reported here, indicate that capping proteins from different sources disrupt microfilament bundles in a variety of cell types causing their disintegration from the distal end towards the centre of the cell.

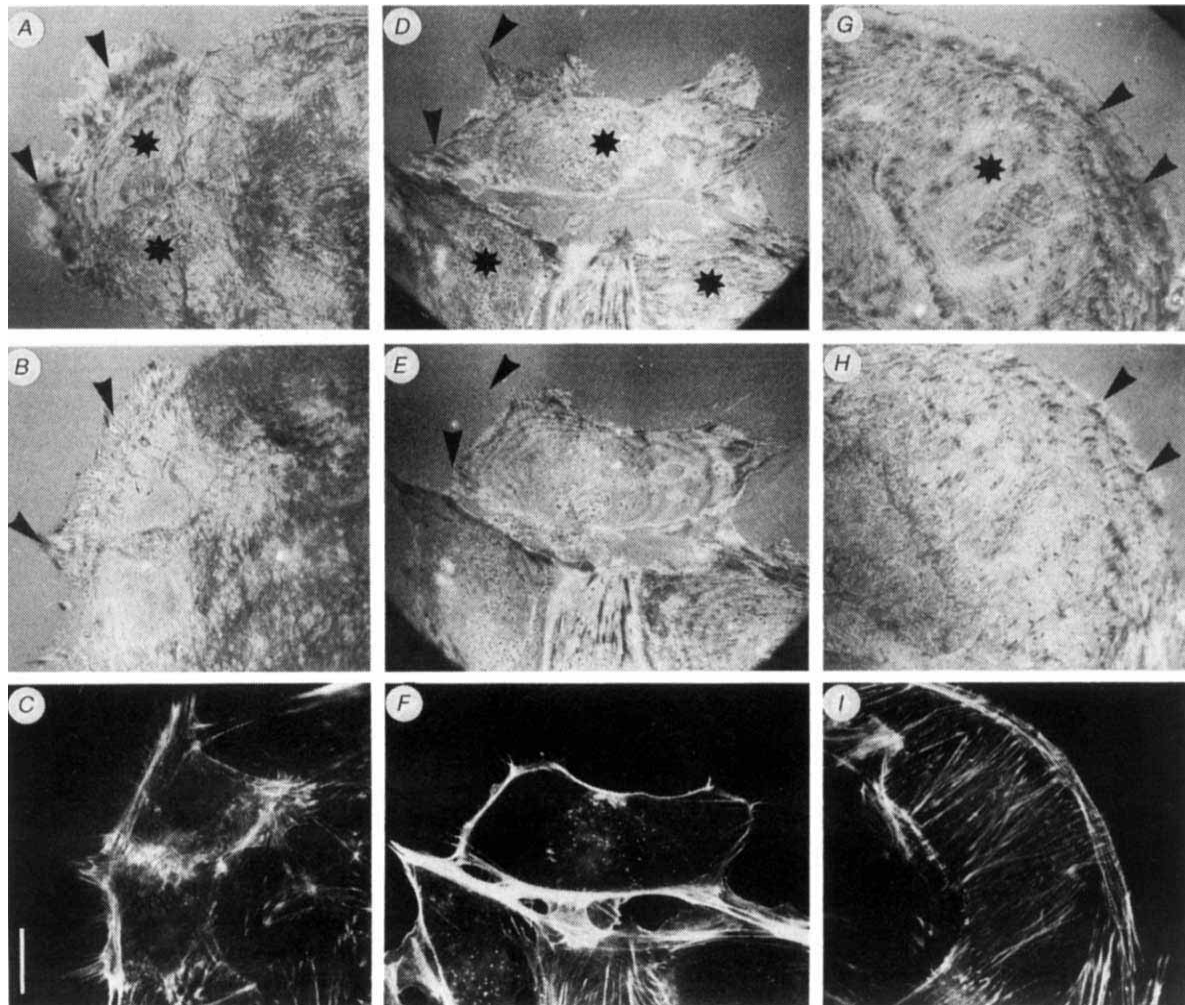


Fig. 1 Effect of microinjection of BCP, PCP or buffer on epithelial cells. PtK₂ cells (asterisks) were injected ($\sim 10^{-7}$ μ l per cell) with: A–C, 0.01 mg ml⁻¹ BCP in BCP buffer (0.02 M imidazole pH 7.2, 0.5 mM dithiothreitol (DTT), 1 mM EGTA, 0.15 M NaCl); D–F, 0.01 mg ml⁻¹ PCP in PCP buffer (0.01 M Tris-HCl pH 7.5, 1 mM DTT, 15% sucrose); G–I, BCP buffer alone. Reflection contrast images of the living cells were photographed immediately before (A, D, G) and 20 min after (B, E, H) injection. Cells were then fixed with 3.7% paraformaldehyde and stained for actin with rhodamine-coupled phalloidin²¹ after 30 (C, I) or 60 (F) min, respectively. While the marginal lamellae of peripheral cells retracted in all three samples (B, E, H), the focal contacts, seen as black dots in the reflection contrast images (arrowheads), as well as the microfilament bundles, remained unaltered in the case of buffer (G, H). In contrast, injection of BCP (A, B) or PCP (D, E) produced a weakening or disappearance of focal contacts (arrows in A, D) paralleled by disintegration of radial microfilament bundles (C, F). Scale bar, 20 μ m.

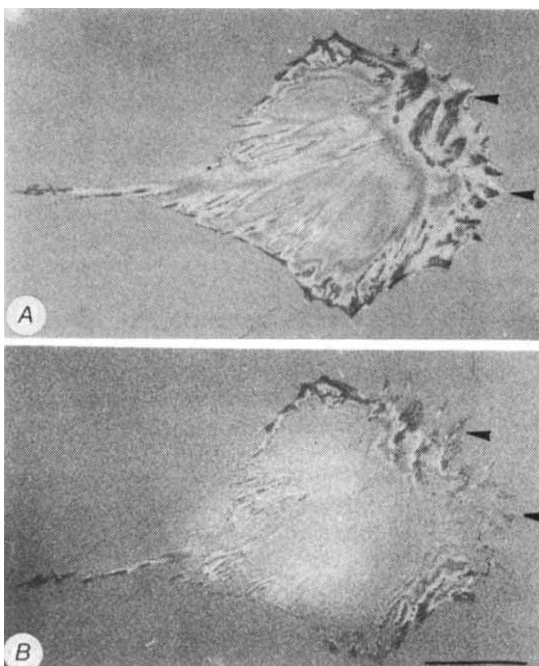


Fig. 2 Effect of microinjection of BPC on focal contacts of newborn rat fibroblasts. Live cells were photographed with reflection contrast microscopy before (A) and 20 min after (B) injection with 0.02 mg ml⁻¹ BCP in BCP buffer. Many of the prominent focal contacts seen in the untreated fibroblast (arrowheads in A) have already disappeared after 20 min (arrowheads in B), but, due to the remaining contacts with the substratum, this cell has not yet contracted, in contrast to the fibroblast shown in Fig. 4A. Scale bar, 20 μ m.

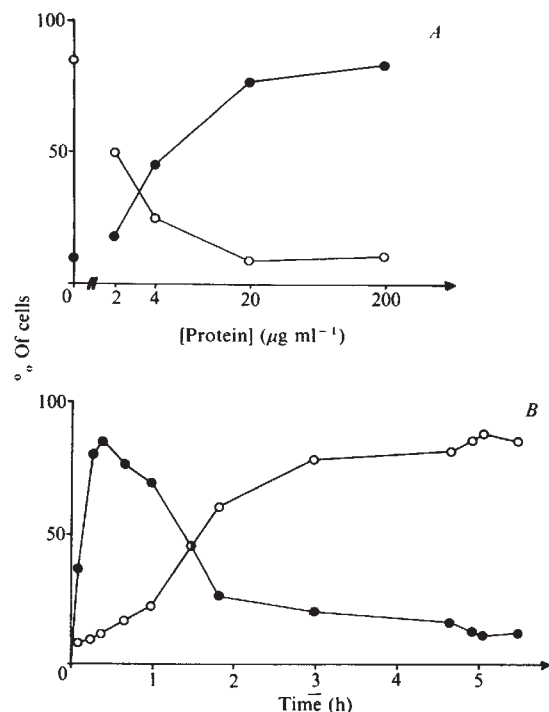


Fig. 3 Substrate adhesion and microfilament organization of PtK₂ cells microinjected with BCP, in relation to the concentration of the injected protein (A), or to the time period after injection (B). Cells were injected and processed for staining with rhodamine-phalloidin as described for Fig. 1. ●, Cells which showed no focal contacts and/or microfilament bundles; ○, cells which showed prominent focal contacts and radial microfilament bundles. The actual numbers of injected cells in each experiment ranged from 30 to 55 cells in A and 98–114 cells in B. In each experiment, a small population of cells fitted into neither of the two categories ('undecided' cells), thus the values do not total 100%. A, Cells injected with BCP buffer alone (control points given on the ordinate), or with increasing concentrations of BCP, were fixed 30 min after injection. The control contains ~15% of cells in the category showing no focal contacts and/or microfilament bundles. These probably represent cells which were killed immediately by being injected, plus the cells not expressing radial microfilament bundles at the time of injection. B, Cells injected with 0.02 mg ml⁻¹ BCP, fixed and examined for focal contacts and/or microfilament bundles at various time points after injection. After 5 h, the cells had fully recovered.

Purification and some biochemical properties of an actin capping protein isolated from mammalian brain (BCP, molecular weight (MW) 63,000) and one from the acellular slime mould *Physarum polycephalum* (PCP, cap-42, MW 42,000), have been described^{10–13}. Both these proteins, when incubated with preformed actin filaments and with actin monomers in substoichiometric amounts, allow further growth of the filaments but restrict it to the slow-growing end^{10–13}. Thus, *in vitro*, they cap the fast-growing end of the filaments, but do not fragment them. When we injected these proteins into living epithelial or fibroblast cells (rat kangaroo PtK₂, primary mouse mammary epithelial cells, human WI 38 or primary mouse fibroblasts) using the Graessmann technique¹⁴, we observed a profound effect on the cells' morphology. When 0.1 mg ml⁻¹ BCP (~10⁻⁷ μl per cell, which corresponds to roughly 2 × 10⁶ molecules per cell), was injected into PtK₂ cells, which grow in patches with strong intercellular adhesions, they reacted by immediately withdrawing their marginal lamellae, followed by weakening or complete disappearance of their substrate adhesion sites (focal contacts), as seen in reflection contrast microscopy, and by a disintegration of their radial microfilament bundles. At the same concentration, both proteins were equipotent (Fig. 1A–C, D–F). The intercellular junctions as well as the marginal, ring-like microfilament bundle frequently expressed in PtK₂ cells seemed unaffected (see Fig.

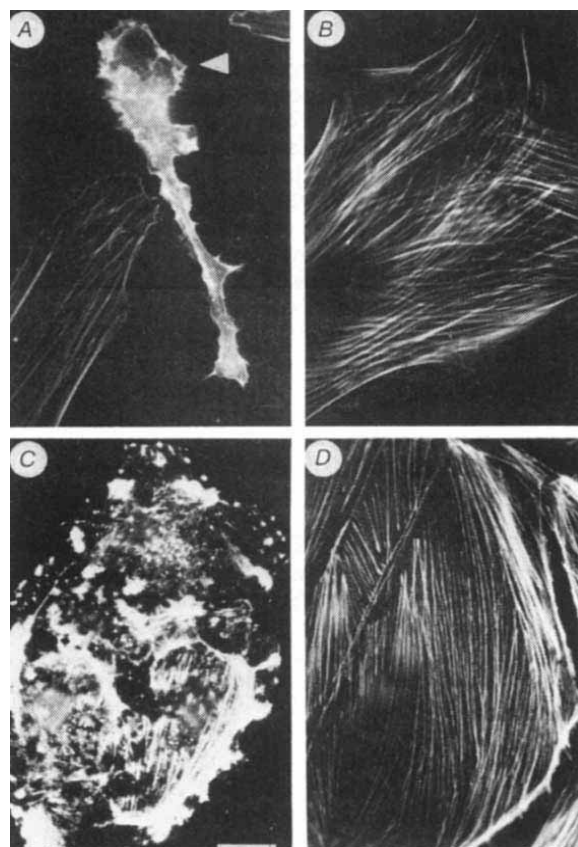


Fig. 4 Effect of BCP and phalloidin on living compared with lysed cells. A, WI 38 human fibroblast (arrowhead) injected with 0.01 mg ml⁻¹ BCP, fixed 30 min after injection; B, mouse fibroblasts treated with the following lysis buffer: 0.1 M morpholinoethane sulphonic acid pH 6.9, 1 mM EGTA, 1 mM guanosine triphosphate, 2 mM MgCl₂, 4% polyethylene glycol 6000, 0.2% Triton X-100, then washed with phosphate-buffered saline, and incubated with 0.1 mg ml⁻¹ BCP for 3 h. C, PtK₂ cells incubated in 1 × 10⁻⁵ M cytochalasin D in tissue culture medium for 5 min; D, PtK₂ cells treated with lysis buffer, washed and then incubated with the same concentration of cytochalasin D as in C. Cells in A were fixed with paraformaldehyde and stained for indirect immunofluorescence with anti-actin antibody²²; cells in B–D were stained with rhodamine-phalloidin.

1C, F). Control cells which were injected with buffer alone, sometimes also retracted their marginal lamellae (Fig. 1G, H), but effects on their focal contacts and microfilament bundles were never observed (Fig. 1G–I).

Fibroblasts showed even more drastic morphological changes after injection of capping proteins. The loss of their prominent focal contacts was visualized in the reflection contrast microscope much more easily than with epithelial cells (Fig. 2). Also, as fibroblasts are not held in position by intercellular junctions, cells contracted dramatically when injected with capping protein (Fig. 4A). Such cells were easily lost during processing for fluorescence microscopy, indicating that their attachment to the substratum had become extremely weak.

Figure 3A shows the expression of focal contacts and radial microfilament bundles of PtK₂ cells as a function of the concentration of capping protein injected. When the kinetics of these morphological changes were followed with cells that had received 0.01 mg ml⁻¹ of either BCP or PCP, it was seen that the distal ends of microfilaments, where they ended in focal contact sites, were affected earlier than the proximal region. The reflection contrast images of adhesion sites in living cells became weaker and then vanished within 5–30 min after injection, indicating a detachment of the cell from the substratum. Simultaneously, as shown by parallel fixation and staining for filamentous actin, the bundling of the distal ends of microfila-

ments within the focal contacts seemed to be affected: the microfilaments 'splayed out'. Over the next 60 min, the radial microfilament bundles disintegrated progressively, until only short lengths remained in the perinuclear area. After that stage, cells began to overcome the effect of the capping proteins: radial microfilament bundles started to grow outwards from the perinuclear area. After 4–5 h, microfilament bundles as well as focal contacts were fully restored (Fig. 3B).

The drastic change in the organization of microfilaments within the region of focal contacts was also observed at the ultrastructural level (not shown). Ultrathin sections of cells which had lost all their focal contacts after injection of high concentrations ($0.05\text{--}0.1\text{ mg ml}^{-1}$) of capping proteins, as seen in reflection contrast microscopy, showed no microfilaments, except for the marginal bundle. Transition stages of this disintegration process could be observed in cells injected with lower concentrations, in which focal contacts had degenerated into a series of small patches, with microfilaments lying in parallel or even in a divergent configuration, instead of the normal convergent one.

In vitro, the capping proteins used here have the same effect on the growth of actin filaments as the fungal alkaloid cytochalasin^{15–18}. As seen in electron micrographs, both agents interfere with the addition of actin monomers to the fast-growing end of S_1 -decorated filaments. In living cells, cytochalasin had been shown previously to destroy microfilament bundles effectively¹⁹, and, even at low concentration, to eliminate focal contacts²⁰. However, the microfilament bundles of lysed cell models had proven stable to the action of cytochalasin B¹⁹. We found the same difference in reactivity of microfilament bundles of living versus lysed cells with respect to capping proteins: microfilament bundles of detergent-treated cells were stable even against high concentrations of BCP (Fig. 4) and PCP (not shown), that is, they were neither fragmented nor dissolved. These findings demonstrate that the capping proteins do not cut (sever) preformed actin filaments in cytoskeletal preparations.

Our results demonstrate that capping proteins from different sources can disrupt the microfilament organization and contact sites of actin filaments in various animal cells, altering the cell's morphology and substrate adhesion. Both capping proteins used here act only on microfilament bundles in live cells, suggesting that the individual actin filaments within such a bundle are in dynamic equilibrium with the actin subunit pool in living cells, and that capping proteins interfere with this equilibrium.

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Switching of subunit composition of muscle spectrin during myogenesis *in vitro*

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Spectrin comprises a family of polypeptides thought to be involved in mediating linkage of actin filaments to the plasma membrane in a wide variety of cell types (for reviews see refs 1–3). Spectrin is present as a tetramer composed of two non-identical subunits^{4–6}. Most cells express a common subunit with a molecular weight (M_r) of 240,000 (240 K; termed α -spectrin)⁷ in association with a polymorphic cell type-specific subunit: M_r 260 K in the intestinal terminal web (termed TW260)⁸, M_r 235 K in nervous tissue^{6,8,9}, liver⁶, lymphocytes¹⁰ and fibroblasts¹¹ (termed γ -spectrin¹⁰; also referred to as fodrin⁹), and M_r 225/220 K in erythrocytes and adult cardiac and skeletal muscle¹² (termed β' - and β -spectrin, respectively). We show here that primary chicken myoblasts express predominantly $\alpha\gamma$ -spectrin, but on terminal differentiation *in vitro* the cells gradually switch to $\alpha\beta$ -spectrin as a result of the onset of β - and β' -spectrin synthesis and by the subsequent differential stabilization of β - and γ -spectrin. This switching correlates with known changes in the biophysical properties and function of the developing muscle sarcolemma and cytoskeleton.

During myogenesis *in vitro*, the subunit composition of muscle spectrin was determined by immunoprecipitation of ³⁵S-methionine-labelled polypeptides with antibodies specific for the α - and β -subunits of chicken erythrocyte spectrin^{7,12}. Although γ -spectrin does not react with either of these antibodies^{7,10,12} it was detected in the immunoprecipitates as a result of the quantitative reassociation of the SDS-solubilized γ -subunit with the α -subunit during incubation with the antibodies (Fig. 1)^{10,12}.

Primary chicken myoblasts (12 h after plating) that were undergoing rapid proliferation in culture (DNA doubling time ~ 14 h) expressed predominantly α - and γ -spectrin in a 1:1 molar ratio as detected by Coomassie blue staining (Fig. 1B). Both spectrin subunits were synthesized at approximately the same rates (Fig. 2A), with turnover rates ($t_{0.5}$) similar to the DNA doubling time (Table 1). β -spectrin was synthesized also in unfused myoblasts (Fig. 1B), although at a rate approximately one-third of either α - or γ -spectrin (Fig. 2A). However, little or no β -spectrin accumulated at this stage so as to be detectable by Coomassie blue staining (Fig. 1B). Following 36 h in culture, when ~50% of the myoblasts had fused to form postmitotic multinucleated myotubes, the total amount of γ -spectrin had decreased relative to that of α -spectrin and small amounts of β -spectrin were detected by Coomassie blue staining, as well as low levels of ³⁵S-methionine incorporation into β' -spectrin (Fig. 1C). After 4 days in culture, immature myotubes synthesized α -, γ -, β' - and β -spectrin at approximately the same rate (Figs 1D, 2B). However, the rates of synthesis of α - and γ -spectrin were approximately one-third of those detected in the primary myoblasts and were now at the same level as that of β -spectrin, which remained constant over the same period (compare Fig. 2A and B). The $t_{0.5}$ of each spectrin subunit had not changed significantly (Table 1). By day 15, the rates of synthesis of α - and β -spectrin in the mature myotubes were at a level similar to those in the 4-day cultures of immature myotubes, while those of γ - and β' -spectrin were one-half to one-third less (compare Fig. 2B and 2C). Analysis of the $t_{0.5}$ of each spectrin subunit revealed that a dramatic change had occurred during myotube maturation (Table 1). The $t_{0.5}$ of α - and β -spectrin had increased approximately tenfold while, during the same period, the $t_{0.5}$ of γ - and β' -spectrin had not

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increased significantly; the $t_{0.5}$ of total proteins increased approximately fourfold (not shown). Figure 1F shows that little or no γ -spectrin was present in 15-day cultures of mature myotubes as detected by Coomassie blue staining.

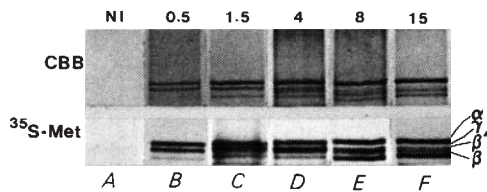


Fig. 1 Subunit composition of spectrin changes during myogenesis. Lane A shows that nonimmune sera did not immunoprecipitate any spectrin subunits detectable by Coomassie blue staining or fluorography. Lanes B–F, immunoprecipitates of muscle spectrin subunits α -, γ -, β' - and β -spectrin with anti- α - and β -spectrin antibodies from 0.5-day (lane B), 1.5-day (C), 4-day (D), 8-day (E) and 15-day (F) cultures of chicken myogenic cells; the upper panel shows the Coomassie blue-stained (CBB) gels and the lower panel the corresponding fluorograms (^{35}S -Met). Several minor protein contaminants were immunoprecipitated also with the nonimmune serum and therefore represent non-specific reactants with the anti- α - and β -spectrin antibodies (not shown). The stoichiometric reassociation of equimolar amounts of α - and γ -spectrin during incubation with anti- α -spectrin antibodies has been demonstrated previously^{10,12}.

Methods: Primary cultures of embryonic chicken thigh muscle were prepared as described elsewhere^{22,23}. For the 0.5- and 1.5-day cultures, the cells were preplated twice²⁴ to remove fibroblasts. This procedure resulted in cultures 90–95% free of contaminating fibroblasts. For the 0.5- and 1.5-day cultures, cells were plated at a density of 6×10^6 cells per 100 mm Petri dish; for the remaining cultures, cells were plated at an initial density of 2×10^6 cells per 100 mm Petri dish. The medium was replaced every 2 days and on days 3–4.5 cytosine arabinofuranoside ($1 \mu\text{M}$ final concentration) was added to selectively kill any dividing cells. Cells were labelled *in vitro* with L- ^{35}S -methionine ($\sim 1,100 \text{ Ci mmol}^{-1}$; NEN) as follows. Each Petri dish was washed twice with 5 ml MEM without methionine and supplemented with 1% heat-inactivated, dialysed horse serum (MEM-Met). The cells of each Petri dish were then incubated at 37°C in 4 ml MEM-Met with $50 \mu\text{Ci } ^{35}\text{S}$ -methionine. At the end of the labelling period (in this case, 1–2.5 h) the cells were processed for immunoprecipitation. In all cases anti- α - and β -spectrin antibodies were combined in the same immunoprecipitates. All subsequent operations were performed at $0-4^\circ\text{C}$ unless otherwise stated. Each plate was washed in Ca^{2+} - and Mg^{2+} -free, Earle's balanced salt solution (CMF/EBSS) and the cells scraped off the dish with a rubber policeman into 5 ml cold CMF/EBSS. The cell suspension was centrifuged at 1,000g in a clinical centrifuge, and the resulting pellet resuspended in 1 ml of 20 mM Tris-HCl pH 7.4, 120 mM NaCl (Tris-saline) and recentrifuged at 12,000g in an Eppendorf microcentrifuge. The cell pellet was dissolved in 100 μl of 1% (w/v) SDS, 5 mM EDTA, 10 mM Tris-HCl pH 7.4, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (buffer A), by boiling for 2 min. The solution was diluted 10-fold with 0.1% (w/v) SDS, 1% (w/v) nonidet-P40, 130 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA (buffer B). To reduce nonspecific binding, samples were precleared by incubation with 5 μl of preimmune rabbit serum and 100 μl of a 10% (w/v) suspension of fixed *Staphylococcus aureus* for 3 h. After centrifugation at 12,000g, the pellet was discarded and 1 μl each of α - and β -spectrin antisera was added to the supernatant. The IgG fraction of each antiserum had been partially purified by fractionation with ammonium sulphate at 50% saturation; the specificity of the antibodies has been described in detail elsewhere^{7,12}. The samples were incubated at 0°C overnight. 100 μl of 10% (w/v) fixed *S. aureus* were added for 2 h, and the immune complex pelleted at 12,000g in an Eppendorf microcentrifuge. The pellet was resuspended in 1 ml of buffer B and recentrifuged; this procedure was repeated three times. The final pellet was resuspended in SDS-sample buffer²⁵ and boiled for 1 min. The staphylococci were pelleted as above and the supernatants applied to a 10% SDS-polyacrylamide gel as described previously^{25,26}. The gels were stained with Coomassie blue, photographed, incubated with Enhance (NEN), then dried and exposed for 1–2 days on Kodak X-omat AR film at -80°C .

These results demonstrate that there is a complete change in the spectrin phenotype during myogenesis *in vitro* from $\alpha\gamma$ -spectrin to $\alpha\beta$ -spectrin, and that this does not occur by the sudden induction of β -spectrin synthesis and concomitant cessation of γ -spectrin synthesis, but rather by a gradual switching of the subunit composition of spectrin during myotube maturation. In order to understand the regulation of the assembly and stabilization of the muscle spectrin complex, we have used the estimated rates of synthesis (R) and turnover ($t_{0.5}$) to predict the ratio of spectrin subunits (x) accumulating at steady state at different stages of myogenesis by using the equation:

$$x = R[(1 - e^{-kt_{0.5}})/k] \text{ where } k = \ln 2/t_{0.5} \quad (1)$$

(Table 1). This analysis shows that primary myoblasts are programmed to start accumulating β -spectrin before cell fusion so that by 36–48 h the predicted ratio of γ - to β -spectrin is $\sim 2.6:1$ (see Fig. 1C). By day 4, twice the amount of ($\gamma + \beta' + \beta$)-spectrin is predicted to accumulate at steady state compared to α -spectrin, indicative of a transient stage in the switching of the spectrin subunit composition. In 15-day-old mature myotubes the ratio of rates of synthesis of α - to ($\gamma + \beta' + \beta$)-spectrin is $\sim 1:2$. However, due to the fact that newly synthesized α - and β -spectrin are much more stable than γ - and β' -spectrin, the cell is predicted to accumulate over the next 10 days predominantly the $\alpha\beta$ -form of spectrin in a molar ratio of approximately 1:1. In adult muscle, we have detected approximately equimolar amounts of α - and β -spectrin and also β' -spectrin¹². β' -spectrin may be required for a membrane specialization of adult muscle tissue that is not present in the myotube *in vitro*, thus this subunit does not accumulate *in vitro*.

Taken together, these results suggest that the transition from the $\alpha\gamma$ -spectrin phenotype to the $\alpha\beta$ -spectrin phenotype during myogenesis is regulated primarily at the post-translational level by the differential stabilization of the spectrin subunits. We hypothesize that the stability of β - and γ -spectrin is regulated by the availability of membrane receptors specific for each of these subunits. Thus, membrane receptors for γ -spectrin, but not β -spectrin, are synthesized in primary myoblasts which results in the assembly of a stable $\alpha\gamma$ -spectrin complex ($t_{0.5} = \text{DNA doubling time}$). However, the synthesis of membrane receptors for β -spectrin during myotube maturation results in the progressive assembly and stabilization of $\alpha\beta$ -spectrin. During the same period the amount of membrane receptor for γ -spectrin may decrease, thus causing newly synthesized, but unassembled, γ -spectrin to be degraded relatively rapidly, as indicated by the relatively short half life of γ -spectrin compared to that of β -spectrin. α -spectrin, on the other hand, does not seem to have any specific role in the switching of γ - and β -spectrin as its rate of synthesis and $t_{0.5}$ are similar initially to those of γ -spectrin in the primary myoblasts and later to those of β -spectrin in the myotube. Support for the hypothesis of membrane receptor-dependent spectrin subunit stabilization comes from recent studies on the assembly of the erythroid spectrin network¹³. During erythropoiesis, the amount of β -spectrin synthesized and assembled onto the membrane regulates the rate of assembly of stoichiometric amounts of α -spectrin. However, the association of β -spectrin with the membrane seems to be limited by the availability of membrane-binding sites. In this case the membrane receptor is probably the transmembrane anion transporter which indirectly binds β -spectrin through their mutual association with ankyrin^{14,15}. α - and β -spectrin subunits that are not bound to the membrane are rapidly degraded in the cytoplasm while those subunits assembled onto the membrane have a relatively long half life. The identity of the membrane receptors for γ - and β -spectrin in muscle cells, however, remains to be determined.

Concomitant with the change in the subunit composition of spectrin in the maturing myotube, there is a gradual reorganization of the spectrin network. Indirect immunofluorescence microscopy reveals that unfused primary myoblasts show strong fluorescence with anti- α -spectrin antibodies (Fig. 3A,B) and,

Fig. 2 Change in the rate of synthesis of spectrin subunits during myogenesis. *A*, 0.5-day myoblasts culture; *B*, 4-day immature myotube culture; *C*, 15-day mature myotube culture. Note that the scales for the amount of ^{35}S -methionine incorporation in *B* and *C* are one-third that in *A*. $\circ$, α -spectrin; $\bullet$, γ -spectrin; $\triangle$, β' -spectrin; $\blacktriangle$, β -spectrin. Chicken myogenic cells were prepared as described in Fig. 1 legend and cultured for 0.5, 4 and 15 days. At each time point, triplicate cultures of cells were labelled *in vitro* for 0.5, 1, 2.5 and 5 h respectively, with $50\ \mu\text{Ci } ^{35}\text{S}$ -methionine in MEM-Met as described in Fig. 1 legend. At the end of the labelling period, the cells were scraped off the Petri dish in CMF/EBSS, centrifuged and washed with Tris-saline as described in Fig. 1 legend. The cell pellet was carefully resuspended in exactly 1 ml Tris-saline and a $50\text{-}\mu\text{l}$ (for total ^{35}S -met incorporation) and $150\text{-}\mu\text{l}$ aliquot (for DNA determination) of the cell suspension were removed and precipitated with 5% (w/v) cold trichloroacetic acid (TCA) with $100\ \mu\text{g}$ bovine serum albumin as carrier (see below). The remaining ($800\ \mu\text{l}$) cell suspension was processed for immunoprecipitation with anti- α - and β -spectrin antibodies and subsequent gel electrophoresis as described in Fig. 1 legend. The Coomassie blue-stained bands corresponding to α -, γ -, β' - and β -spectrin were excised from the gel and incubated overnight at room temperature in $500\ \mu\text{l}$ of 0.1% SDS, $100\ \text{mM}$ NH_4HCO_3 ; $5\ \text{ml}$ Aquasol-2 (NEN) were added and the amount of radioactivity determined in a Beckman LS-233 liquid scintillation spectrometer. To determine ^{35}S -methionine incorporation into total protein the precipitate from the $50\text{-}\mu\text{l}$ aliquot from each cell suspension was placed on a Whatman GF/C filter, washed successively with $10\ \text{ml}$ 5% (w/v) TCA and $10\ \text{ml}$ 95% ethanol, dried and counted in a scintillation spectrometer in $10\ \text{ml}$ Aquasol-2. For DNA determination, the $150\text{-}\mu\text{l}$ aliquot was applied to a GF/C filter, and the precipitate was washed successively with $10\ \text{ml}$ 7.5% TCA, $10\ \text{ml}$ 1 M HCl and $10\ \text{ml}$ 95% ethanol and dried under a lamp. The filters were incubated with $250\ \mu\text{l}$ of 2 M 3,5-diaminobenzoic acid (Aldrich Chemical Co., Milwaukee, Wisconsin) at 65°C for 40 min in a capped scintillation vial; $5\ \text{ml}$ of 1 M HCl were then added to each vial. After allowing glass fibre particles to settle, the solution was transferred to a glass tube, excited at $410\ \text{nm}$ and read at $510\ \text{nm}$ in a Perkin-Elmer fluorescence spectrophotometer, MPF-4 (refs 27, 28). The amount of DNA was determined from a calf thymus DNA standard curve prepared simultaneously. ^{35}S -methionine incorporation was normalized to the DNA values. An average of the triplicate cultures was used for each time point (the variation between each Petri dish of a triplicate was routinely $<15\%$; not shown).

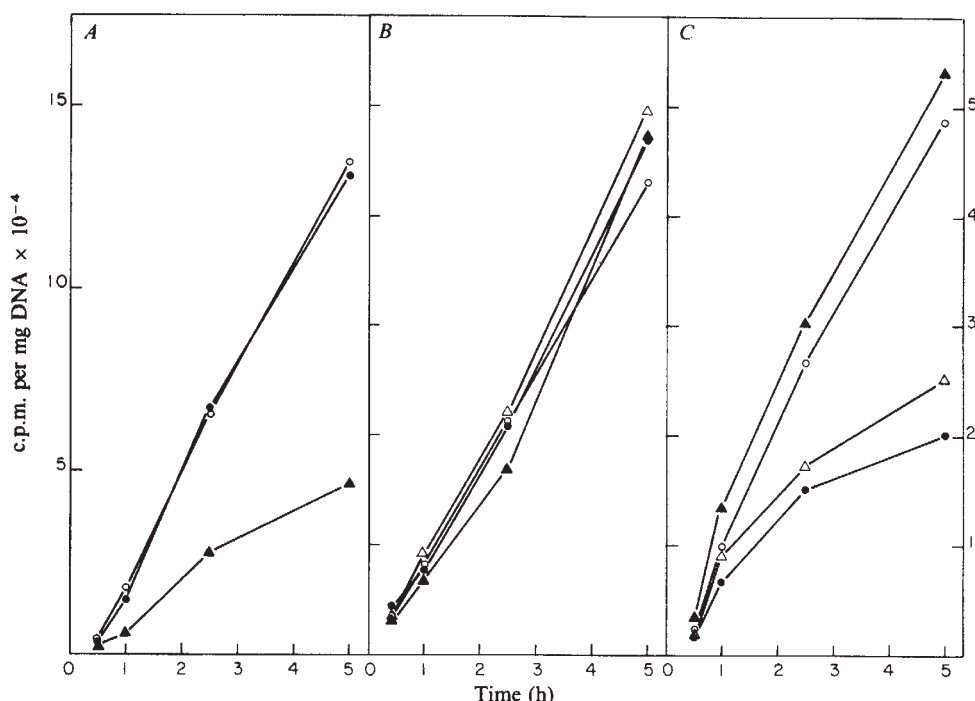


Table 1 Turnover rates, observed ratios of rates of synthesis of different spectrin subunits and predicted ratios of amounts of different spectrin subunits at steady state during myogenesis *in vitro*

		α	γ	β'	β
	$t_{0.5}$	14 h	10 h	—	11 h
12 h myoblasts	Observed ratio of the rates of synthesis (R)	3	3	—	1
	Predicted ratio of amount of each subunit at steady state	3.6	2.6	—	1
		(45 h)	(34 h)		(37 h)
4-day myotubes	$t_{0.5}$	12 h/34 h	11 h/19 h	11 h/17 h	12 h/16 h
	(R)	1	1	1	1
	Predicted ratio	1.8	1.1	1	1.1
		(110 h)	(64 h)	(57 h)	(53 h)
15-day myotubes	$t_{0.5}$	176 h	29 h	25 h	106 h
	(R)	2	1	1	2
	Predicted ratio	11.1	1	1	9
		(>10 days)	(95 h)	(83 h)	(>10 days)

Triplicate cultures of cells were labelled with ^{35}S -methionine in minimal essential medium (MEM)-Met for 2 h as described in Fig. 1 legend. At the end of the labelling period the medium was removed and the cells were washed twice in complete MEM supplemented with 5% horse serum and then each triplicate was incubated for 1, 2.5, 5, 10 and 24 h, respectively, at 37°C . At each time point the cells were scraped off the Petri dish in CMF/EBSS (see Fig. 1 legend), centrifuged and washed in Tris-saline; the final cell pellet was processed for immunoprecipitation and DNA determination as described in Fig. 2 legend. The incorporation of ^{35}S -methionine into each spectrin subunit was determined and then normalized to the DNA values as detailed in Fig. 2 legend. Values of $t_{0.5}$ were estimated by regression analysis of the data points assuming exponential decay kinetics in the case of the 12-h myoblast culture and the 15-day myotube culture. The $t_{0.5}$ values for the 4-day myotube spectrin subunits were estimated assuming biphasic decay kinetics from the early data points (1–5 h) and later data points (5–24 h) respectively, which results in two $t_{0.5}$ values for each subunit. The rates of synthesis (R) were calculated from the data presented in Fig. 2 and are given as a ratio. The predicted steady-state ratio of spectrin subunits (x) was calculated from the $t_{0.5}$ and R of each subunit using the equation $x = R[(1 - e^{-kt_{0.5}})/k]$ where $k = \ln 2/t_{0.5}$. The long-term (steady-state) amount of spectrin subunit can therefore be approximated by the quantity R/k , on the assumption that these rates remain constant during the time period required to reach steady state. The approximate time required in culture to reach 90% of the predicted steady values is given in parentheses. The predicted values for the 4-day myotube spectrin subunits were estimated using the $t_{0.5}$ from the 5 h–24 h data points of the pulse-chase experiment. In all cases the ratios are expressed relative to the spectrin subunit with the smallest absolute value.

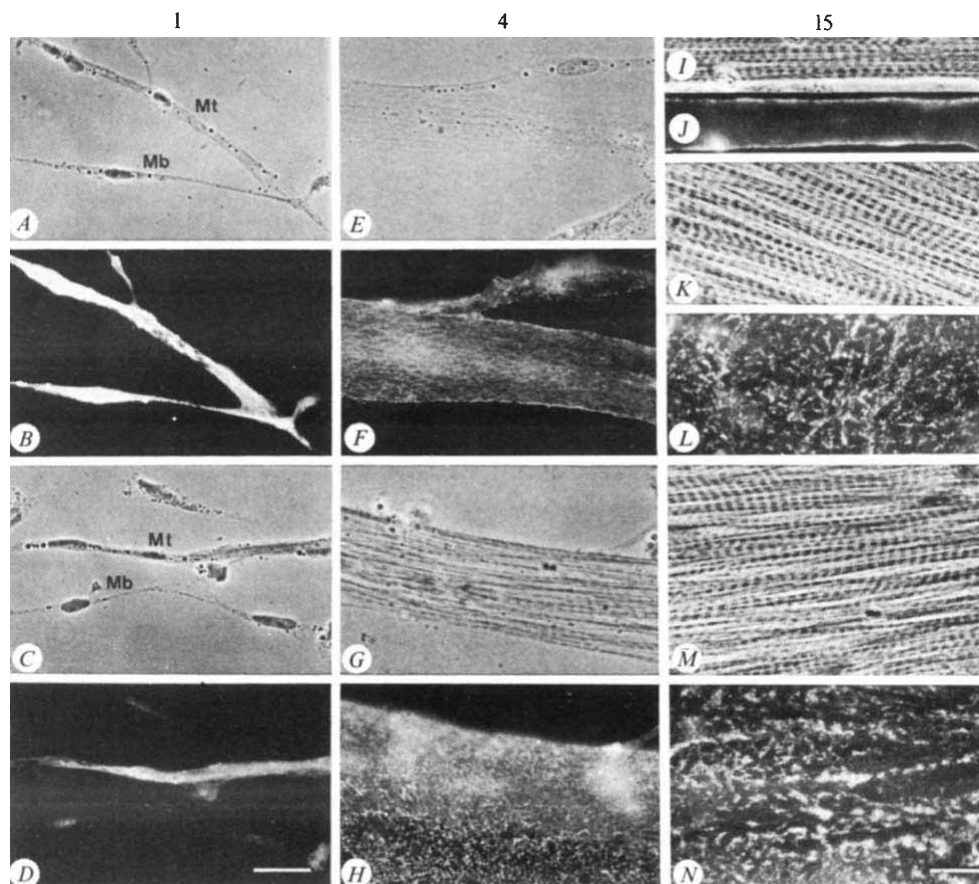


Fig. 3 Redistribuition of spectrin during myogenesis. Micrographs show phase-contrast and corresponding fluorescence images of a 1-day myotube/myoblast culture stained with anti- α -spectrin (A, B) and anti- β -spectrin (C, D) antibodies (Mb, myoblasts; Mt, myotube); a 4-day immature myotube culture stained with anti- α -spectrin (E, F) and anti- β -spectrin (G, H) antibodies; a 15-day mature myotube culture stained with anti- α -spectrin (I–L) and anti- β -spectrin (M, N) antibodies. Scale bars: A–D, 20 μ m and E–N, 13 μ m. The phase-contrast images of 15-day myotubes were photographed in a different plane of focus than the fluorescence images in order to show the cross-striations of the sarcomere. Note that the nonimmune sera gave negative staining^{7,12} and that similar results to those shown above were obtained with anti- α - and β -spectrin antibodies if the first permeabilization step was omitted (not shown); however, negative staining was obtained if Triton X-100 was omitted from the whole procedure (not shown).

Methods: For indirect immunofluorescence, cultures of myogenic cells were grown on collagen-coated coverslips. After 1, 4 and 15 days in culture, coverslips were removed and the cells permeabilized in phosphate-buffered saline (PBS) containing 5 mM MgCl₂, 0.5% (w/v) Triton X-100 for 15 s at room temperature and then fixed at 37 °C for 10 min in PBS, 1.75% (v/v) formaldehyde. The coverslips were then rinsed in PBS, 0.5% (w/v) Triton X-100 for 10 min at room temperature. Duplicate coverslips were incubated with either anti- α - or β -spectrin antibody diluted 1:10 in PBS, 0.5% (w/v) Triton X-100 for 30 min at 37 °C in a humidified atmosphere. The coverslips were briefly rinsed as above and then incubated with fluorescein-conjugated goat anti-rabbit IgG (Miles-Yeda) diluted 1:150 in PBS, 0.5% (w/v) Triton X-100 for 30 min at 37 °C. Coverslips were rinsed briefly as above, mounted in Elvanol²⁹, and photographed with Kodak Tri-X film with a Leitz phase/epifluorescence microscope.

as expected, little or no fluorescence with anti- β -spectrin antibodies (Fig. 3C,D). Immature myotubes in the same culture show a diffuse pattern of fluorescence with both anti- α - and β -spectrin antibodies (Fig. 3A–D). In 4-day-old myotubes, the distribution of α - and β -spectrin has reorganized into a fine, grid-like lattice (Fig. 3F, H). By 15 days, elements of this lattice appear to have coalesced to form patches that are linked together parallel to the direction of the cross-striations of the sarcomere (Fig. 3L, N). This reorganization of the spectrin network appears to be correlated with the gradual accumulation of $\alpha\beta$ -spectrin in the myotube. A similar effect has been observed as spectrin accumulates in erythroid cells during erythropoiesis and is thought to result in a decrease in the mobility of proteins in the plane of the erythrocyte membrane¹⁶. In this respect, it is interesting to note that there is also a gradual decrease in the fluidity of the muscle plasma membrane during myogenesis^{17,18} as assayed by the mobility of acetylcholine receptors¹⁹.

The distribution of $\alpha\beta$ -spectrin in a 15-day-old myotube is not so well developed as that in the adult skeletal muscle cell *in situ*, where the spectrin network appears to aggregate further to form rings around the sarcolemma that coincide with the

regular cross-striations of the Z-lines¹². Several electron microscope studies have demonstrated that the Z-line is attached to the sarcolemma by filaments (for examples, see ref. 20), which are probably part of the cytoskeletal scaffold that integrates individual Z-disks to form a composite Z-line²¹. Thus, the function of spectrin and its gradual reorganization during development may be to mediate linkage of the Z-disk scaffold to the sarcolemma, which would help to maintain the structural integrity of the muscle cell during contraction and relaxation.

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Exon duplication and divergence in the human preproglucagon gene

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Glucagon is a 29-amino acid pancreatic hormone which counteracts the blood glucose-lowering action of insulin by stimulating hepatic glycogenolysis and gluconeogenesis¹. The structure of the hamster pancreatic glucagon precursor has recently been determined from the sequence of a cloned cDNA². Hamster preproglucagon is a 180-amino acid protein which contains five functional regions; a signal or pre-peptide, an NH₂-terminal peptide (also called glucin-related pancreatic peptide, GRPP), glucagon, and two carboxy-terminal glucagon-like peptides (GLP-1 and GLP-2). The sequences of two non-allelic anglerfish pancreatic glucagon precursors^{3–5} have also been determined and their organization is similar but not identical to the hamster protein; they lack the polypeptide segment corresponding to hamster GLP-2. The presence of three regions possessing internal homology, that is, glucagon, GLP-1 and GLP-2, within proglucagon, and the absence of GLP-2 in the anglerfish precursors suggests that the structure of the preproglucagon gene might provide insight into the evolution of this polypeptide. We have isolated and sequenced the human preproglucagon gene and report here that the organization of the human precursor deduced from this sequence is identical to the hamster protein. The gene contains at least three intervening sequences which divide the protein-coding portion of the gene into four regions corresponding to the signal peptide and part of the NH₂-terminal peptide, the remainder of the NH₂-terminal peptide and glucagon, GLP-1, and GLP-2. The data suggest that triplication and subsequent sequence divergence of an exon encoding glucagon or a glucagon-like peptide produced this polypeptide precursor.

A 992-base pair (bp) fragment from the Syrian hamster preproglucagon cDNA², which included the 540-bp coding region, and 28 bp of the 5'-untranslated region and 424 bp of the 3'-untranslated region, was hybridized to *Eco*RI digests of human and hamster DNA using conditions which stabilize mismatched hybrids (Fig. 1). Hamster *Eco*RI fragments of 8,000, 4,600 and 2,100 bp gave intense signals and a 8,700-bp fragment gave a much weaker signal. Human *Eco*RI fragments of 8,800, 5,500 and 2,100 bp showed hybridization.

The 'partial *Hae*III-*Alu*I' human DNA library in bacteriophage λ of Lawn *et al.*⁶ was screened in these hybridization conditions. Two of the 625,000 phage hybridized, and restric-

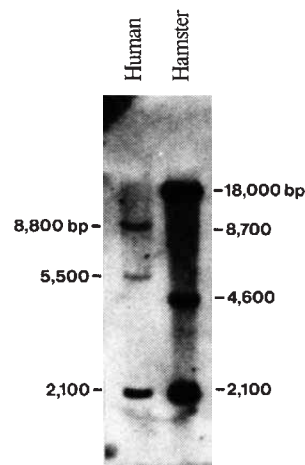


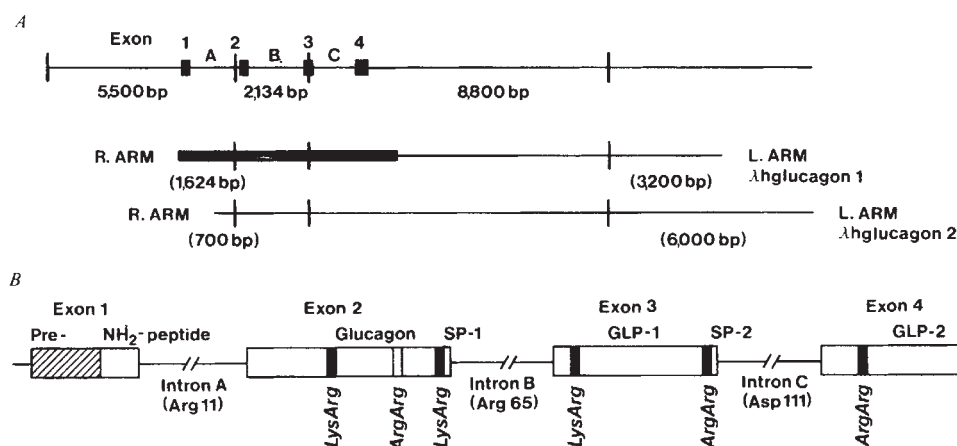
Fig. 1 Hybridization of a hamster cDNA fragment to *Eco*RI digests of human and hamster DNA. The sizes of the hybridizing fragments are indicated. Human placental or Syrian hamster DNA (prepared from transformed B-cells)³¹ was digested with *Eco*RI and fragments >1,000 bp were resolved on a 1% agarose gel. The DNA was transferred to a nitrocellulose filter³² then hybridized to a ³²P-labelled³³ 992-bp *Dde*I fragment of pshglu1 in 50% formamide, 5 × SSC, 10% dextran sulphate, 20 mM sodium phosphate pH 6.5, 5 × Denhardt's solution, 100 μg ml⁻¹ sonicated, denatured salmon testes DNA, and 0.1% SDS³⁴ at 30 °C. The filters were washed for 1 h at 50 °C in 1 M NaCl³⁵ before autoradiography. The hamster lane contained approximately twice as much DNA as the human.

tion mapping indicated that they contained overlapping segments of human DNA (Fig. 2A). Both phage contained *Eco*RI fragments of 2,100 and 8,800 bp which hybridized with the hamster cDNA and thus correspond to two of the hybridizing fragments observed in digests of human DNA. The 1,600 bp *Eco*RI fragment of λ hglucagon 1 also hybridized with the cDNA probe and to a 5,500-bp *Eco*RI fragment of human DNA (data not shown). The 700-bp *Eco*RI fragment of λ hglucagon 2 did not hybridize with the hamster cDNA. The data suggest that there is a single proglucagon gene in the human genome and most of it is included in λ hglucagon 1.

A 6,455-bp segment of λ hglucagon 1 (Fig. 2A) was sequenced and the regions encoding human preproglucagon mRNA were identified by their homology with the hamster cDNA sequence. Three introns of 1,572, 1,668 and 1,369 bp interrupt the protein-coding region of the gene (Figs 2, 3). The four exons encode segments of preproglucagon which correspond to discrete functional regions of the protein, except for the NH₂-terminal peptide which is encoded by exons 1 and 2. Introns B and C interrupt the spacer oligopeptides which separate glucagon and GLP-1, and GLP-1 and GLP-2. Consequently, glucagon, GLP-1 and GLP-2 are each encoded by separate exons as is the signal peptide. Exon 4 may also encode part or all of the 3'-untranslated region of the mRNA.

As the sequence of human preproglucagon mRNA is unknown, the 5' and 3' boundaries of the gene cannot be precisely defined. The 103 nucleotides of the 5'-untranslated region of hamster preproglucagon mRNA could form 14 different alignments with the 162 bp preceding the protein-coding region of the human gene. The homology was low and varied from 30.1 to 35.8%. This region of the human gene contains three possible 'TATA-like' sequences⁷, TATAATC, AATAAAA and TTATAAAA, each followed by a possible cap site approximately 30 bp downstream. Because of the A+T-rich character of this region, the functional significance of these sequences is unknown. Moreover, none of these sequences apparently functions as a promoter in a HeLa cell-free transcription extract using the 'run-off' assay (ref. 8 and L. Rall, unpublished). This region also contains a reasonable splice acceptor site⁹, TTCTGTGTTCTGACAG, beginning 10 bp upstream from the start of the protein-coding segment of exon 1.

Fig. 2 Structure of the human preproglucagon gene. **A**, Linkage map of the preproglucagon gene region. A composite map of this region is indicated as are the structures of the two phage. *EcoRI* sites are indicated by vertical lines, and the sizes of the *EcoRI* fragments are given. The numbers in parentheses indicate that these subfragments have an *EcoRI* linker at one end. The solid boxes indicate the positions of the regions encoding preproglucagon. There are several copies of dispersed middle-repetitive sequences in the 8,800-bp *EcoRI* fragment distal to the gene and in the 3,200- and 6,000-bp fragments. The region of Δ hglucagon 1 sequenced is indicated by the thick black line. **B**, Schematic representation of preproglucagon indicating the regions encoded by each exon. The polypeptide regions of the precursor are indicated. The cross-hatched region is the pre- or signal peptide. SP, spacer peptide. The pairs of basic amino acids are indicated and solid boxes represent cleavage sites. The amino acid whose codon is split by the intron is indicated.



We believe that most of the 5'-untranslated portion of human preproglucagon mRNA may be encoded by another exon. In the 3' region of the human gene, the segment from nucleotide 6,313 to 6,455 possesses 77.6% homology with the 3'-end of the hamster cDNA and also contains a polyadenylation signal and may represent the 3'-end of the human gene. Thus, unless there is an intron in this region, the 3'-untranslated portion of the mRNA would be at least 1,143 bases which is unusually long; this portion of the hamster mRNA is 475 bases long.

Glucagon and glucagon-containing polypeptides are synthesized in the endocrine pancreas, intestine, brain and possibly the salivary gland (as well as the stomach in the case of the dog)¹⁰⁻¹³, the pancreas representing the most abundant source of glucagon. The amounts of glucagon-containing polypeptides in the intestine, the next most abundant source, are 1% of those of the pancreas. As pancreatic preproglucagon² contains the sequences of several intestinal glucagon-containing peptides^{14,15} including glucagon-immunoreactant-1 (GLI-1) or glicentin, which corresponds to pancreatic proglucagon(1-69), it has been suggested² that tissue-specific processing of a common precursor could generate the array of peptides observed in the pancreas and intestine. The presence of only a single gene encoding proglucagon supports this notion. Two of the polypeptides which could arise from processing of proglucagon, GLP-1 [proglucagon(72-108)] and GLP-2 [proglucagon(126-159)], are cryptic as they have not been isolated and their physiological properties are unknown. We have compared human and hamster preproglucagon in order to assess the importance of the two cryptic peptides as well as other regions of the precursor (Fig. 3, Table 1). The two precursors are of a similar size, that is, 179 and 180 amino acids; hamster preproglucagon has an additional basic amino acid at its carboxy-terminus. The amino acid sequences of human and hamster proglucagon are highly conserved (94.3% homology). In fact, the homology is greater than that observed between human and hamster proinsulin (84.9%) (Fig. 3, Table 1). The homology within preproglucagon varies from 65.0 to 100%, the pre-peptide being the most variable region. The glucagon moieties are identical as expected because the sequences of all mammalian glucagons that have been described are the same¹⁶. The homology between the NH₂-terminal peptide, GLP-1 and GLP-2 regions is much greater than for the C-peptides of proinsulin and is similar to that between the insulin A and B chains. The sequence conservation suggests that the NH₂-terminal peptide and GLP-1 and GLP-2 may possess unique biological properties and represent undescribed hormones. The non-glucagon segments of the precursor could also have a structural role in the processing of glucagon from the precursor.

In contrast to mammals which synthesize a single pancreatic glucagon precursor of 179-180 amino acids, anglerfish produces

Table 1 Comparison of human and hamster preproglucagon and preproinsulin

Region	Homology	
	Amino acid	Nucleotide
Preproglucagon	163/179 (91.1%)	472/537 (87.9%)
Proglucagon	150/159 (94.3%)	428/477 (89.7%)
Pre-peptide	13/20 (65.0%)	44/60 (73.3%)
NH ₂ -peptide*	27/32 (84.4%)	83/96 (86.5%)
Glucagon	29/29 (100.0%)	80/87 (92.0%)
Spacer peptide 1*	10/10 (100.0%)	28/30 (93.3%)
GLP-1	37/37 (100.0%)	101/111 (91.0%)
Spacer peptide 2*	16/17 (94.1%)	47/51 (92.2%)
GLP-2	31/34 (91.2%)	89/102 (87.2%)
Preproinsulin†	91/110 (82.7%)	269/330 (81.5%)
Proinsulin	73/86 (84.9%)	213/258 (82.6%)
Pre-peptide	18/24 (75.0%)	56/72 (77.8%)
B chain	29/30 (96.7%)	78/90 (86.7%)
C-peptide*	24/35 (68.6%)	77/105 (73.3%)
A chain	20/21 (95.2%)	58/63 (92.0%)

* Includes the pair(s) of basic amino acids.

† The amino acid sequence of hamster preproinsulin in the one-letter code (G.I.B., in preparation) is:

MTLWMRLPLLLTLVLWEPNPAQAFVNQHLGSHLVEAL
YLVCGERGFFYTPKSRRGVEDPQVAQLELGGGPGADDL
QTLALEVAQQKRGIVDQCCTICSLSLYQLENYCN.

The sequence of human preproinsulin is from Bell *et al.*³⁰

two non-allelic pancreatic precursors of 122 and 124 amino acids³⁻⁵. The organization of the two anglerfish preproglucagons is similar to the human and hamster proteins except that they lack the second spacer peptide and a region corresponding to GLP-2, both of which are encoded by exon 4 (see Fig. 2 and discussion in ref. 2). Since intron-exon organization is generally conserved during evolution^{17,18}, exon 4 is either absent or not expressed in these anglerfish genes. Interestingly, the carboxy-terminus of human proglucagon would be identical to the anglerfish proteins, that is, Arg-Arg-Glu, if intron C were not removed since there is an in-frame translation stop codon adjacent to the 5'-end of the intron (Fig. 3) (an in-frame stop codon in this position is also present in introns A and B). However, we have no evidence for alternative splicing of the mRNA precursor, or for other forms of preproglucagon in the mammalian pancreas.

The organization of the human preproglucagon gene suggests that tandem duplication, in either one or two steps, of an exon encoding glucagon or a glucagon-like protein has occurred. The addition of an exon encoding the signal peptide, acquisition of signals for removing the introns, and subsequent divergence of the amplified exons probably produced this gene. The evolution of the proglucagon gene is different from that of other

polypeptide hormones which also contain internal homologous units. For example, the melanocyte-stimulating hormones of pro-opiomelanocortin^{19,20}, and the enkephalins of pro-enkephalin²¹ are encoded by a single exon. However, the signal peptide, and the promoter and cap site of these genes are each exon-encoded. The process which produced the preproglucagon gene may be a variation of that which generated the globin^{22,23}, human growth hormone²⁴⁻²⁶ and leukocyte interferon^{27,28} gene families, where tandem duplications of a gene and flanking sequences occurred. However, the unit of duplication in the preproglucagon gene was probably an exon and flanking sequence.

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The structure of the human preproglucagon gene supports the notion of Gilbert²⁹ that the mosaic structure of eukaryotic genes reflects their evolutionary history and that new proteins can be produced by reassortment and amplification of exons of existing proteins.

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Linkage of human apolipoproteins A-I and C-III genes

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It has recently been suggested that polymorphisms in the human apolipoprotein A-I (apo A-I) gene locus may be related to the development of premature atherosclerosis¹ and hypertriglyceridaemia². To understand if and how these polymorphisms affect apo A-I gene expression, we studied the genomic sequences flanking the apo A-I gene. Here we show the presence of another apolipoprotein gene, apolipoprotein C-III (apo C-III), approximately 2.6 kilobases (kb) downstream of the 3' end of the apo A-I gene. We also show that the apo A-I and apo C-III genes are convergently transcribed and that a polymorphism previously reported to be associated with hypertriglyceridaemia² may be due to a single base pair substitution in the 3'-noncoding region of apo C-III mRNA.

Based on previous analysis of protein sequence data, it had been suggested that the genes for apo A-I and apo C-III were derived by gene duplication from a common evolutionary precursor³. In addition, we have observed that a polymorphism in the apo A-I gene correlated with a deficiency of both apo A-I and apo C-III in patients with premature atherosclerosis¹. Subsequent work has shown that this polymorphism is due to a DNA insertion of at least 7.5 kb in one of the exons of the apo A-I gene, and, interestingly, genomic blotting analysis shows no major differences in the apo C-III gene between these patients and normal individuals⁴. We therefore speculated that a close linkage may exist between the apo A-I and apo C-III genes. To examine this possibility, it was necessary to isolate apo C-III double-stranded (ds)-cDNA clones to use these as molecular probes for genomic blotting analysis. An apo C-III ds-cDNA clone (pCIII-606) was isolated from a human liver cDNA library⁵ using a synthetically prepared oligonucleotide probe (Fig. 1). DNA sequencing of the insert of pCIII-606 showed that it extends from codons 58 to 79 (C-terminus) of

apo C-III mRNA. The insert also contains the entire 3'-noncoding region of apo C-III mRNA and a part of the poly(A) tail (Fig. 1). The amino acid sequence predicted from the DNA sequence agrees with the previously reported^{6,7} apo C-III amino acid sequence. Genomic mapping analysis of the human apo C-III gene was carried out by digesting human DNA with various restriction enzymes, blotting and hybridization with the ³²P-labelled insert of pCIII-606 (pCIII-606 probe). Figure 2A shows the resulting autoradiogram from such genomic mapping analysis. The sizes of the hybridization bands were determined and a restriction map of the human apo C-III gene was constructed (Fig. 2B).

We have previously isolated and characterized a genomic clone (λ apo A-I 6) which contains the entire apo A-I gene⁸. To determine whether apo A-I and apo C-III genes are closely linked, λ apo A-I 6 DNA was digested with various restriction enzymes, blotted and hybridized with the pCIII-606 probe. The resulting autoradiogram (Fig. 3A) shows that the λ apo A-I 6 clone carries DNA sequences that are strongly homologous to the apo C-III cDNA probe. The sizes of these hybridization bands were determined and a restriction map of λ apo A-I 6 was constructed (Fig. 3B). Comparison of Figs 2A and 3A shows that the genomic clone λ apo A-I 6 contains an approximately 16-kb insert, part of which carries the apo C-III gene.

LysAspLysPheSerGluPheTrpAspLeuAspProGluValArgProThrSerAlaValAlaAla
AAGGACAAGTCTCTCTGAGTTCTGGGATTGGACCCCTGAGGTCAGACCAACTTCAGCCGTGGCTGCCTGA
↓
GACCTCAATACCCCAAGTCCACCTGCCTATCCATCCTGGAGCTCCTGGGTCCTGCAATCTCCAGGGC
SacI
TGCCCTCTAGGTTGCTTAAAGGACAGTATTCTCAGTCTCTCTACCCACCTCATGCTGGCCGC
CCTCCAGGATGCTGGCTCCCAATAAGCTGCACAAGAAGCTGCGpoly(A)

Fig. 1 Nucleotide sequence of the insert in clone pCIII-606. The sequence is displayed in the 5' to 3' direction. The portion of the DNA sequence coding for apo C-III is indicated by the amino acid sequence above it. The termination codon and polyadenylation signal are boxed. The arrow shows the nucleotide involved in the SacI restriction site polymorphism (see text). The sequence corresponding to the synthetically prepared oligonucleotide probe for cDNA library screening is underlined. This probe consisted of a mixture of 14mers to account for the degeneracy of the code. Synthesis of this mixture of 14mers, screening of the cDNA library, and DNA sequence analysis were performed as we described elsewhere²⁵.

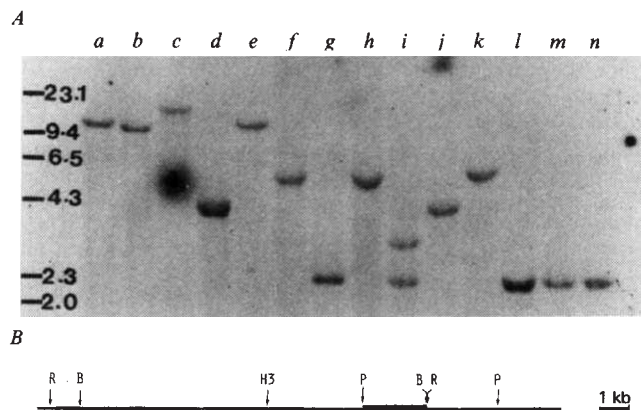


Fig. 2 Genomic mapping of the human apo C-III gene. **A**, DNA prepared from human blood¹ was digested with restriction enzymes and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized with ³²P-labelled (nick-translated) insert of clone pCIII-606 (pCIII-606 probe). The autoradiogram of the filter is shown. The restriction enzymes used were *Eco*RI (*a*), *Bam*HI (*b*), *Hind*III (*c*), *Pst*I (*d*), *Eco*RI plus *Bam*HI (*e*), *Eco*RI plus *Hind*III (*f*), *Eco*RI plus *Pst*I (*g*), *Bam*HI plus *Hind*III (*h*), *Bam*HI plus *Pst*I (*i*), *Hind*III plus *Pst*I (*j*), *Bam*HI plus *Eco*RI plus *Hind*III (*k*), *Eco*RI plus *Bam*HI plus *Pst*I (*l*), *Eco*RI plus *Hind*III plus *Pst*I (*m*), *Bam*HI plus *Pst*I (*n*). Size markers were *Hind*III-digested λ DNA (Biolabs). The sizes (kb) of these markers are indicated on the left of the autoradiogram. (Note the double band in lane *i* is due to partial *Bam*HI digestion.) **B**, Restriction map of the apo C-III gene in the human genome. This map was constructed using the results obtained in **A**. The thick line indicates the region hybridizing to the pCIII-606 probe. Restriction sites are indicated as follows: *Eco*RI (R), *Bam*HI (B), *Pst*I (P) and *Hind*III (H3).

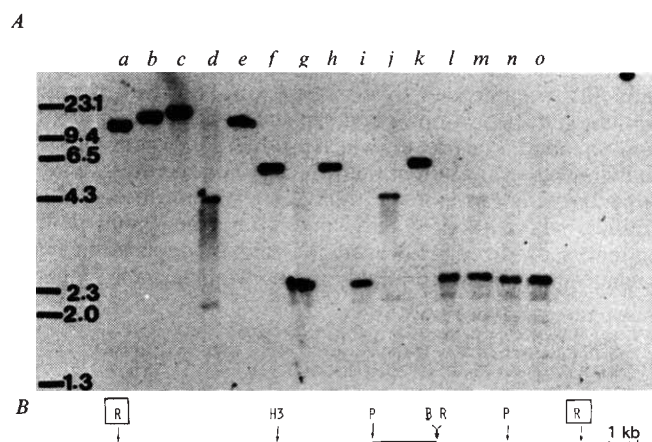


Fig. 3 Restriction analysis of clone λ apo A-I 6. **A**, DNA prepared from clone λ apo A-I 6 (1 μ g per lane) was digested with restriction enzymes. Restriction digests in each lane are as follows: lanes *a-n* contain the same enzymatic digestions as the respective lanes in Fig. 2. Lane *o* is *Eco*RI + *Bam*HI + *Hind*III + *Pst*I digestion. The products were electrophoresed on a 1% agarose gel, blotted onto nitrocellulose filters, and hybridized with ³²P-labelled pCIII-606 probe. The resulting autoradiogram is shown. The size markers are as in Fig. 2. **B**, Restriction map of λ apo A-I 6. This map was constructed using the results obtained in **A**. The thick line indicates the region hybridizing to the pCIII-606 probe. Restriction sites are indicated as in Fig. 2B. The *Eco*RI sites due to the Charon 4A λ vector are enclosed in boxes.



Fig. 4 Linkage map of human apo A-I and apo C-III genes. Thick lines represent apo A-I and apo C-III genes as indicated. Arrows beneath the thick lines indicate the direction of transcription of each gene. The dotted arrow shows the location of the *Sac*I restriction site polymorphism (see text). Restriction sites are indicated as in Fig. 2B and the *Sac*I restriction site is represented by S.

We therefore conclude that apo A-I and apo C-III genes are closely linked in the human genome and that clone λ apo A-I 6 contains both genes. DNA sequencing and restriction mapping analyses (data not shown) of λ apo A-I 6 DNA allowed the construction of a restriction map of the region of the genome containing the linked genes (Fig. 4). These analyses also indicated that the genes are separated by ~2.6 kb and are convergently transcribed, as shown in Fig. 4.

These results support the previous suggestion³ that the apo A-I and apo C-III genes may be evolutionarily related. Furthermore, they indicate that these apolipoprotein genes may be coordinately regulated or share common controls of gene expression. In support of this speculation, we point out that in patients with severe premature atherosclerosis an insertion in the apo A-I gene has resulted in complete absence of both apo A-I and apo C-III gene products¹⁹. Clearly, more work is needed to establish if, and at what level, such a functional linkage of the apo A-I and apo C-III genes may occur.

Finally, DNA sequence analysis of the region of clone λ apo A-I 6 which contains the sequence corresponding to the 3'-noncoding segment of apo C-III mRNA indicated the presence of the nucleotide C 40 base pairs (bp) downstream from the TGA terminator of apo C-III gene (data not shown). The nucleotide sequence obtained from clone pCIII-606, however, indicates the presence of the nucleotide G at exactly the same position (Fig. 1). It is interesting that this transversal mutation involves the 5'-end nucleotide of the recognition sequence for the restriction enzyme *Sac*I (GAGCTC). Figure 4 shows the position at which this polymorphism occurs with respect to apo A-I and apo C-III genes, and indicates that the presence or absence of this polymorphic *Sac*I site would produce, in genomic blotting analyses using an apo A-I probe, 3.2- and 4.2-kb hybridization bands, respectively. Genomic blotting analysis of individuals heterozygous for this *Sac*I site polymorphism should produce both 3.2- and 4.2-kb hybridization bands when an apo A-I probe is used. Using an apo A-I probe, Rees *et al.* have recently identified this polymorphism and

suggested that homozygosity for the 3.2-kb hybridization band is related to hypertriglyceridaemia². Our data now allow us to conclude that this polymorphism may be due to a C-G to G-C transversion in the 3'-noncoding region of the apo C-III mRNA which creates a new *Sac*I restriction site.

Apo C-III is a major constituent of triglyceride-rich lipoproteins^{10,11}, and has been shown to be present in increased concentration in the plasma of hypertriglyceridaemic individuals^{12,13}. *In vitro*, apo C-III has been shown to inhibit the activities of lipoprotein lipase^{14,15}, the rate-limiting enzyme in the clearance of triglyceride-rich lipoproteins and hepatic lipase¹⁶. Apo C-III has also been shown to decrease the uptake of lymph chylomicrons (triglyceride-rich) by the perfused rat liver¹⁷⁻¹⁹. If these mechanisms operate *in vivo*, primary abnormalities in apo C-III resulting in excessive plasma apo C-III levels could have an aetiological role in hypertriglyceridaemia by diminishing the catabolism of triglyceride-rich lipoproteins. In fact, hypertriglyceridaemic subjects in some studies have shown an increased ratio of plasma apo C-III to apo C-II (another apolipoprotein)²⁰⁻²⁴, and such patients in other studies have shown a diminished fractional catabolic rate of triglyceride-rich lipoproteins¹². We therefore conclude that if plasma apo C-III levels play a part in the aetiology of hypertriglyceridaemia and if the association between hypertriglyceridaemia and the polymorphic *Sac*I site holds, the present demonstration of the genetic lesion responsible for this polymorphism may provide an insight into the regulation of apolipoprotein gene expression of importance in understanding human hypertriglyceridaemia.

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Encoding of both subunits of ribulose-1,5-bisphosphate carboxylase by organelle genome of *Cyanophora paradoxa*

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The intracellular photosynthetic entities (cyanelles) of the protozoan *Cyanophora paradoxa* (Fig. 1) have long been regarded as endosymbiotic cyanobacteria due to morphological and physiological similarities such as unstacked thylakoids¹, a rudimentary yet lysozyme-sensitive cell wall^{2,3}, and phycobilisomes as light-collecting apparatus⁴. However, recent investigations of cyanelle DNA cast doubt on this classification. The cyanelle genome has been found to resemble chloroplast DNA in size and structure^{5–7}. In light of the endosymbiont hypothesis of eukaryotic cell evolution⁸ these paradoxical features of the cyanelles suggest that *C. paradoxa* is an evolutionary intermediate between cyanobacteria, the presumed plastidal ancestors, and modern photosynthetic organelles. The ribulose-1,5-bisphosphate carboxylase (RuBPCase) of *C. paradoxa* is composed of eight large (LSU) and eight small (SSU) subunits⁹. In higher plants and green algae the LSU of the enzyme is encoded by plastid DNA and the SSU is nucleus-encoded¹⁰. In cyanobacteria the LSU gene has been located on the chromosome¹¹. No data are available on the SSU gene. Here we have investigated the cyanelles' evolutionary position by determining the location of the genes for the two subunits of RuBPCase in *C. paradoxa*. By heterologous hybridization the genes for both subunits of RuBPCase have been located on the cyanelle genome. Furthermore, LSU and SSU probes exhibit homology to the same *Bgl*II and *Hind*III fragment, suggesting a close proximity of the two genes.

Methods were developed that allowed for the isolation of nuclear and cyanelle DNA without significant cross contamination. Cyanelles were isolated from frozen cell pellets. On thawing, 90% of the host cells were disrupted. This amount increased to 99% during the following four or five washes in 5×TE buffer (1×TE buffer is 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), which also freed the cyanelles of host cell debris. Centrifugations were at 1,000g for 15 min at 4 °C. After resuspension of the cyanelle pellet in 1×TE buffer, lysis was brought about by adding 250 mM EDTA, pH 8.0 and 25% SDS to final concentrations of 50 mM and 1%, respectively. After a 2 h incubation at 37 °C in the presence of 0.5 mg ml⁻¹ nuclease-free proteinase K (BRL), the lysate was subjected to density gradient centrifugation to remove residual contamination by nuclear DNA (1 ml of 10 mg ml⁻¹ ethidium bromide and 7.0 g of CsCl were added per 6.5 ml lysate). Centrifugation was at 40,000 r.p.m. for 36 h at 20 °C in a Beckman Ti75 rotor.

For the isolation of nuclear DNA a fresh cell pellet (unfrozen) was resuspended in 1×TE buffer and made 1% in Triton X-100. This treatment selectively lysed the host cell without any apparent effect on the cyanelles, which could be removed by centrifugation. The supernatant was incubated for 30 min at 37 °C with 0.5 mg ml⁻¹ proteinase K and extracted once with 5×TE buffer-saturated phenol and once with chloroform/isoamylalcohol [24:1 (v/v)] to remove bulk protein. Nuclear DNA was purified by banding in CsCl gradients in the presence of ethidium bromide as described above.

A 580-base-pair (bp) DNA fragment from maize chloroplasts¹² and a 1-kbp DNA fragment from *Chlamydomonas* chloroplasts¹³ (as modified by R. Hallick, personal communication from R. Haselkorn) were used as probes to locate the LSU gene in *C. paradoxa*. The *Chlamydomonas* probe represents the 3' two-thirds of the LSU coding region. The maize fragment contains the presumed active site region from within the LSU coding region. Neither probe fragment exhibited any homology

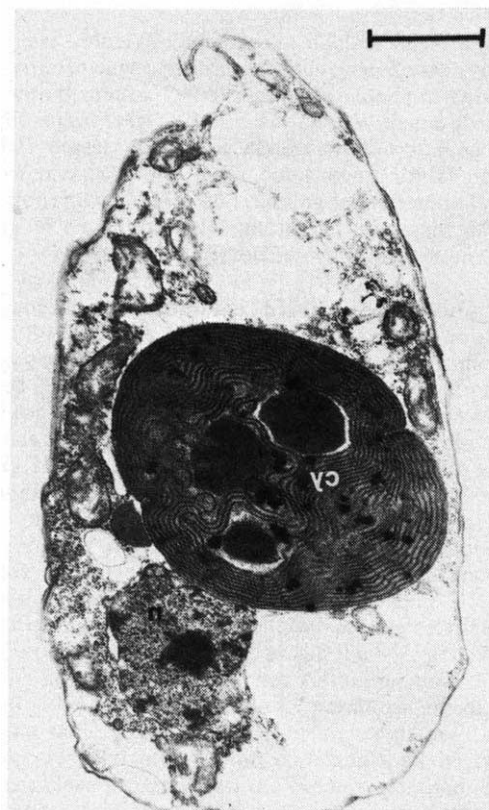


Fig. 1 Transmission electron micrograph of *Cyanophora paradoxa*. Fixations were for 2 h in 1% glutaraldehyde, followed by 1 h in 2% OsO₄, all at 4 °C. cy, Cyanelle; n, nucleus. Scale bar, 1 μm.

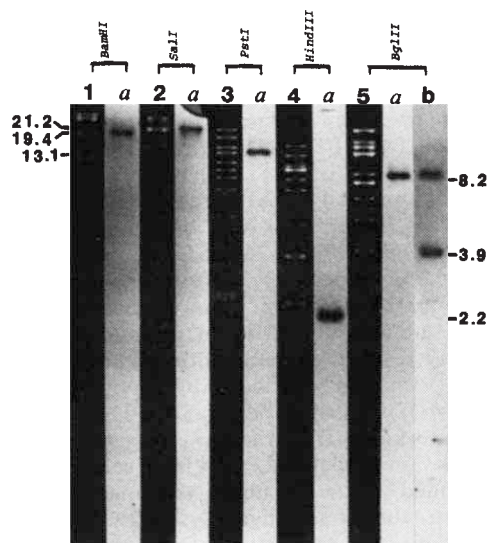


Fig. 2 Hybridization of *C. paradoxa* cyanelle DNA to probes for the LSU gene of RuBPCase. Cyanelle DNA was digested with *Bam*HI, *Sal*I, *Pst*I, *Hind*III and *Bgl*II (lanes 1–5, respectively). Fragments were separated on a 1% agarose gel and transferred to nitrocellulose¹⁴. Lanes 1–5 show the ethidium bromide stained gels. Lanes 1a–5a and 5b show respective autoradiograms. Blots were hybridized to the *Pst*I insert from pZmc 461 (ref. 12) (lanes 1a–5a) and to the *Hind*III insert from pCr 34.1 (ref. 13) (lane 5b) labelled with ³²P by nick translation. A NEN-Nick-translation kit ([α -³²P]dCTP) was used. Hybridizations were performed at 60°C for 24 h in 6×SSC buffer, 1×Denhardt solution¹⁷, 100 μ g ml⁻¹ of calf thymus DNA. The blots were washed twice in 3×SSC buffer, 0.1% SDS and twice in 1×SSC buffer, 0.1% SDS at room temperature. The numbers indicate the sizes (in kbp) of the cyanelle DNA fragments homologous to the LSU probes.

to Southern¹⁴ blots of *C. paradoxa* nuclear DNA digests (data not shown). However, probing various cyanelle DNA restriction digests with the maize DNA fragment yielded one hybridization band per digest (Fig. 2, lanes 1a–5a). With the exception of the *Bgl*II digest, the same results were obtained with the *Chlamydomonas* probe (data not shown). *Bgl*II possesses a recognition site within the LSU coding sequence in *C. paradoxa*, resulting in an additional homologous fragment of approximately 3.9 kbp that was detected with the *Chlamydomonas* probe (Fig. 2, lane 5b).

The gene for the RuBPCase SSU in *C. paradoxa* was located using a 280-bp DNA fragment from pea as the probe¹⁵. The stringency conditions were lowered and the probe concentration increased (1×10^7 c.p.m. per lane) in the hybridization to compensate for the presumed low degree of homology between the SSU genes from these distantly related organisms. In a cyanelle DNA *Bgl*II digest, the same 8.2-kbp fragment that was found to be homologous to the two LSU probes also hybridized to the SSU probe (Fig. 3a, lane 4). Similar results were obtained when a *Hind*III digest of cyanelle DNA was probed for the SSU gene. Again, the 2.2-kbp fragment that bears the LSU gene was homologous to the SSU probe (Fig. 3b, lane 1). Considering the position of the LSU probes relative to each other, it is possible tentatively to assign the SSU gene a position on the 5' side of the LSU gene. Assuming that the coding regions for the SSU and LSU in *C. paradoxa* are of approximately the same size as those from higher plants (0.368 and 1.425 kbp, respectively^{15,16}), we conclude that the two genes are in very close proximity on the cyanelle genome. This gene arrangement could allow a concerted regulation of expression that would ensure the synthesis of both subunits in equimolar amounts. Using the described conditions, no homology could be detected between the SSU probe and nuclear DNA digests (Fig. 3a, lanes 1–3; Fig. 3b, lanes 2, 3). This result remained unchanged even when the amount of digested nuclear DNA

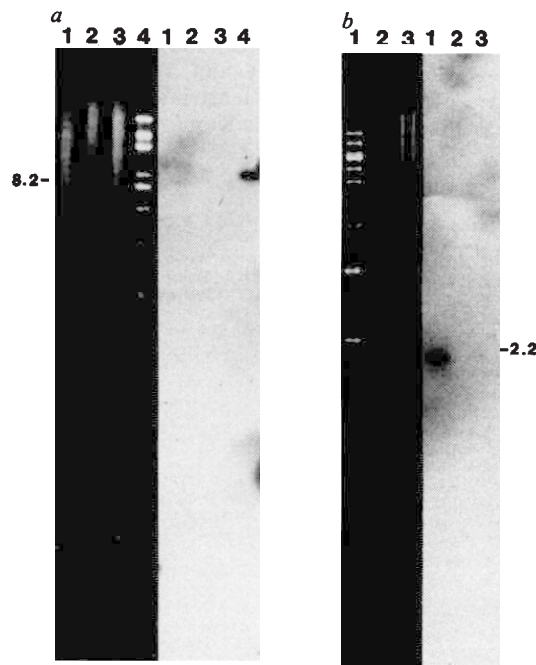


Fig. 3 Hybridization of *C. paradoxa* nuclear and cyanelle DNA to a probe for the SSU gene of RuBPCase. Digests of nuclear DNA with *Bam*HI (a, lane 1), *Hind*III (lane a2, b2), *Eco*RI (lanes a3, b3) and of cyanelle DNA with *Bgl*II (lane a4) and *Hind*III (lane b1) were separated on 1% agarose gels and blotted onto nitrocellulose filters¹⁴. The left sides of a and b show the ethidium bromide stained gels, the right sides show the respective autoradiograms after hybridization of the blots to the labelled *Hind*III insert of pSSU 160 (ref. 15). The hybridization conditions were as described in Fig. 1 legend. Two washes in 6×SSC buffer, 0.1% SDS were followed by two washes in 3×SSC buffer, 0.1% SDS, all at room temperature.

was increased to 10 μ g per lane. These data suggest that the SSU gene has not been duplicated and transferred to the nucleus.

The location of the genes for both subunits of RuBPCase on the cyanelle genome of *C. paradoxa* is unexpected considering the strong structural similarity between a plastid and cyanelle DNA^{5–7}. However, in spite of the limited coding capacity of the latter, cyanelles should not be regarded as mere photosynthetic organelles because they seem to have retained a greater degree of independence than have plastids. Taking into account the cyanelles' morphological similarities to unicellular cyanobacteria, the present results favour the concept of cyanelles as an intermediate stage between prokaryotic endosymbionts and photosynthetic cell organelles. A more precise classification of *C. paradoxa*, however, especially concerning the LSU and SSU genes in cyanobacteria and red algae, seems premature without further data.

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Evidence for earlier date of Ubeidiya, Israel hominid site

REPENNING AND FEJFAR¹ have suggested a late Pliocene age for the occurrence of an early Acheulian tool industry at Ubeidiya, Israel. This was done by correlating the vertebrate fauna associated with the implements to similar faunas in Europe, in particular with the fauna at Seneze, France. Reassigning the age of the Ubeidiya fauna led to assumptions concerning the age of the faunal transition from the Villanyan to the Biharian Micro-Mammal zone of Europe.

Repenning and Fejfar stated they found no substantial reason for considering the Ubeidiya fauna younger than 2 Myr. We emphasize that the arguments for assigning the late Villafranchian Seneze fauna below the Olduvai Subchron are equivocal. Therefore, the data do not force the Ubeidiya fauna into the Pliocene; indeed, because of the uncertainty of the placement of the Biharian-Villanyan boundary, the Acheulian tools at Ubeidiya can easily be younger than the Acheulian industry at Olduvai Gorge.

Thus, we support the conclusions of Repenning and Fejfar that the Ubeidiya fauna is older than previously believed, and that it is most similar to the late Villafranchian, Seneze, and Kislang faunas. However, we object to their inference that the Villanyan-Biharian boundary can be placed unambiguously at 1.9 Myr.

Figure 1 of Repenning and Fejfar implies that this transition has been securely placed within the magnetic-polarity time scale (MPTS), and occurs below the Olduvai Subchron. Unfortunately, this is not certain, and on this note, we review the evidence for the age of this boundary and the age of the Seneze fauna.

The Seneze fauna occurs at the top of a series of lacustrine sediments which are younger than 2.3 Myr as reported by Prevot and Dalrymple². Prevot reported information on the magnetic stratigraphy of these sediments and noted a normally magnetized subchron in the upper part of the section below the fossils. Unfortunately, the palaeomagnetic data are incomplete with no thermal demagnetization performed on the sedimentary section. Therefore, there are ambiguities in the placement of the fossils in the MPTS. First, the reported normally magnetized subchron may not be real, since the laboratory work does not convincingly demonstrate the recovery of the depositional polarity. Second, even if it was real, it is not possible to correlate it unambiguously with the palaeomagnetic time scale. It could represent either one of the Reunion Subchrons or the Olduvai. In any case this would differ from the practice of French workers who usually place the fossil horizon above the Olduvai Subchron³.

Since Repenning and Fejfar believe that the fauna at Ubeidiya is close to the Villanyan-Biharian boundary, it is worthwhile to review the evidence for placement of the boundary in the MPTS. The Biharian is judged by these authors to begin with the first appearance of *Allophaiomys*. The earliest occurrence of this mammal within a sequence correlated to the MPTS, even indirectly, is in the Brielle fauna of the Netherlands, where it occurs in sediments of Eburonian age above the Olduvai Subchron⁴. Van Montfrans⁵ places the Eburonian above the Olduvai. Although a palaeomagnetic study was not done on the sediments containing the fossils, the correlation being made on the basis of pollen stratigraphy, it is most likely that the Villanyan-Biharian boundary is younger than the Olduvai Subchron.

We have studied palaeomagnetic samples from Ubeidiya (N.D.O.) collected by one of us (G.K.). All samples were found reversely magnetized and therefore older than the 730,000-yr old Brunhes-Matuyama boundary.

Kukla⁶ reported the occurrence of the late Villafranchian rodent *Mimomys pliogenicus* in the middle of the Krems loess sequence of southern Czechoslovakia within the stratigraphical interval KR7, KR8, and KR9, correlated with the upper Matuyama Chron (about 1.0 Myr) in the local magnetic reversal sequence. And, underlying strata, KR10, still in the Matuyama Chron yielded another late Villafranchian rodent, *Mimomys hungaricus*. Both *Mimomys pliogenicus* and *Mimomys hungaricus* are recorded from the Villany fauna of southern Hungary (type Villanyan)⁷ and are characteristic of the latest Villafranchian mammal zone, MN17⁸. The Seneze fauna of France and the Brielle fauna of Holland are most recently correlated⁸ with the younger part of mammal zone MN17. Therefore, latest Villafranchian—and the Ubeidiya fauna—could be considered as young as 1.0 Myr.

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REPENNING REPLIES—Opdyke *et al.* question the identity and the existence of the brief normal event slightly down section from the Seneze fauna and they question the time of the beginning of the Biharian micromammal age in Europe. I am not qualified to discuss the reality of the short normal event in the Seneze section reported by Prevot and Dalrymple¹ other than to say that the event to which they assign it, with subsequent correction of polarity event names², and the age they thus infer for the fauna is entirely consistent with the biochronology of microtine rodents of the world. The youngest lava flow below the lake beds filling the volcanic crater at Seneze is in the vicinity of 2.3-Myr old, the overlying lake beds are reversely magnetized and reasonably assigned to the early part of the Matuyama Chron, lacustrine deposition within a crater is reasonably considered to have been continuous in the lack of evidence to the contrary, and if a short section of normal polarity, confirmed by three samples, is found in these lake beds it is reasonable to presume that it represents the older of the two polarity events now assigned to the Reunion Subchron (then called the Olduvai). This scenario is strengthened by varve counts in the original report¹. All interpretation of evidence should be subject to question, but question seems pointless until conflicting evidence is found that suggests another interpretation may be closer to the truth.

The question of the age of the Villanyan-Biharian boundary requires a bit more discussion; for brevity we did not discuss this at length in our letter³. The definition of these and other mammalian age terms are and should be constantly growing as more is learned about the faunas on which they are based. We stated³ that we were using the terms as used and defined by Fejfar and Heinrich⁴ in 1981 and I would like to emphasize that this usage is more refined, extended geographically, and defensible than as originally defined by Miklos Kretzoi in 1941⁵. By our definition, Villanyan and Biharian are defined on the basis of microtine ('arvicolid') rodents; other mammals may be assigned to these ages only by association with appropriate microtine rodents. Fejfar and Heinrich list 16 microtine species as occurring in the oldest Biharian faunas; many of these are not restricted to the Biharian age. In brief, the beginning of the Biharian age is marked by the first appearance of abundant microtines with rootless cheek teeth; most Villanyan microtines have cheek teeth with roots. The same definition is applied to the oldest Irvingtonian faunas

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in North America and these faunas appear at exactly the same time in both areas. In discussion with Opdyke, I have used the genus *Allophaiomys* as an example because it appears in abundance in both Biharian and Irvingtonian faunas; its cheek teeth are rootless and its earliest records in North America are above a 2.0-Myr ash in Kansas and during the Olduvai Subchron in Saskatchewan. This is in explanation of the statements of Opdyke *et al.*; *Allophaiomys* was not mentioned in our letter³. As mentioned by Opdyke *et al.*, *Allophaiomys* occurs in the Brielle boring of The Netherlands⁶ and this fauna was mentioned in our letter.

But the Brielle boring contains two faunas. *Allophaiomys* occurs at a depth of 57–58 m and is assumed to be associated with an Eburonian flora⁶: a record that has impressed Opdyke *et al.* The rootless microtine *Dicrostonyx* occurs in the Brielle boring at a depth of 65–66 m and is associated with a Tiglian C-6 flora; *Dicrostonyx* also first occurs in the earliest Biharian faunas of Europe⁴. The fauna from the Egypte channel in The Netherlands⁷ contains only microtines with rooted cheek teeth, is associated with a Tiglian C-5 flora, and is channeled into beds with a Tiglian C-4 flora⁷; the Egypte channel fauna is a Villanyian fauna. Thus the Villanyian–Biharian boundary is rather closely tied to either the lowest Tiglian C-6 or the highest Tiglian C-5. Tiglian C-6 is oldest Olduvai Subchron and Tiglian C-5 is basal Olduvai and pre-Olduvai reversely magnetized^{8,9}. The base of the Olduvai is close to 1.9-Myr old and so is the Villanyian–Biharian boundary in Europe. However, we did show³ the base of the Olduvai a bit too young on our Fig. 1 according to current refinements of the age of KBS Tuff in East Africa.

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Disruptive or directional selection?

AN elegant experiment on larval competition using unmarked, unselected strains of *Drosophila melanogaster* has been described by Pérez-Tomé and Toro¹. Vials containing 10 unrelated full-sibships produced more adult flies than did vials containing 10 full-sibships related as half-

sibs through a common father. The authors interpret this result as indicating that larvae with relatively similar genotypes compete with each other more directly than do larvae with relatively dissimilar genotypes, and they suggest that this supports the view that significant amounts of genetic variation are maintained in nature by frequency-dependent disruptive selection arising from differential resource utilization by different genotypes within the same local population. Here we point out that there is another possible interpretation of their results. On this interpretation, selection is strictly directional.

The larvae were extremely crowded. If a female laid approximately one egg per h, the average egg-to-adult survival in this experiment was less than 20%. The population was derived from a large number of ancestors that had been collected from the wild in September 1981. Thus, it seems likely that the population contained substantial genetic variation for characters affecting larval survival in crowded conditions on artificial medium. If such variation was present, the results presented by Pérez-Tomé and Toro can be explained without invoking genotype-specific interactions between larvae, or differential utilization of niches. For simplicity, consider a single locus at which there is an unconditionally favorable allele *A* at frequency *p*. The expected frequency of *A* among the eggs in any vial is always *p*, because parents are always selected at random. But the variance of *p*, over vials, is greater in the 'homogeneous series', where there is only one father per vial, than it is in the 'heterogeneous series', where each of the 10 full-sibships has a different father. (In the multi-locus case, the variance of $\bar{p}$ is also greater in the homogeneous series than in the heterogeneous series.)

By assumption, the number of adults emerging from a vial is an increasing function of the actual frequency of *A* among the eggs laid in that particular vial. But suppose this number obeys a law of diminishing returns, due to the limited amount of food and space available. It follows that the mean number of adults emerging should be greater for vials in the heterogeneous series than for those in the homogeneous series, because more adults are lost from vials in which the actual frequency of *A* is below *p*, than are gained from vials in which the frequency of *A* is above *p*. This is also expected on the disruptive-selection model, of course, and was the main experimental finding (Table 1 of ref. 1). The directional-selection model makes the additional prediction that the variance of the number of adults emerging per vial should be greater for vials in the homogeneous series than for those in the heterogeneous series. This difference is not demanded by the disruptive-selection model, but might easily appear in a differential niche-utilization

version of it. The difference does appear in the data ($F(120, 120) = 3.5$ $P < 0.001$, for the overall variances in Table 1 of ref. 1).

It seems entirely possible that disruptive selection (of the kind discussed by Pérez-Tomé and Toro) and directional selection (of the kind discussed here) both occurred in this experiment. We do not see how the directional-selection effect can be ruled out, in any experiment in which there is genetic variance between vials within series.

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TORO AND PÉREZ-TOMÉ REPLY—The alternative explanation suggested by Fowler and Seger assumes: (1) the existence of an unconditionally favourable allele *A*, affecting larval survival; (2) the number of adults emerging from a vial is an increasing function of the actual frequency, following a law of diminishing returns. Nevertheless, we fail to see how this law can be accomplished in a system of strict density-dependent selection, as diminishing returns would generally imply frequency-dependent selection. The convexity of the function $f(w, p)$ would only arise if $w_{ij,k} \neq w_{ij,k}$ (where $w_{ij,k}$ is the fitness of the *ij*th genotype in the *k*th family. This is the case of frequency-dependent selection either by genotype-specific interactions between larvae or by differential utilization of niches. But in the usual density-dependent model¹ $w_{ij} = v_{ij}/(1 + a_{ij}N)$ (where v = viability, v/a = carrying capacity, N = number of eggs per vial) and there is no way for the convexity to arise.

A density-dependent selection model would only mimic frequency dependence when $w_{ij} = v_{ij}/(1 + a_{ij}\bar{v})$ (where $\bar{v}$ = family mean egg-larvae viability). This would imply the existence of genes of large effect on viability or more exactly on the non-density-dependent component of fitness. In any case, this last possibility could be ruled out almost completely if our results can be repeated by using larvae directly instead of fecundated females. This experiment is now in progress.

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Genetics at first sight

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A Feeling for the Organism: The Life and Work of Barbara McClintock.
By Evelyn Fox Keller. W. H. Freeman: 1983. Pp.235. \$14.95, £12.95.

IN THE preface, Evelyn Fox Keller describes her book as both biography and intellectual history. In the case of the geneticist Barbara McClintock the two aspects would be difficult to deal with separately; rarely can there have been a life so single-mindedly devoted to scientific investigation and to ideas. The author, a mathematician, sociologist of science and former molecular biologist, has spent much time in conversation both with McClintock herself and with many of her contemporaries and followers. The result is a lively, perceptive and very well written biography, and a stimulating if contentious intellectual history.

After a somewhat rebellious girlhood and a wayward but successful college career, Barbara McClintock quickly made her mark as a cytologist of exceptional flair. As a member of R.A. Emerson's maize genetics group at Cornell (where she was associated with G.W. Beadle and, most importantly, Marcus Rhoades) she obtained elegant and compelling evidence for the equivalence of linkage groups, inferred from genetical data, and chromosomes, directly visible under the microscope. Later, with L.J. Stadler at the University of Missouri, she turned her attention to chromosome breakage and the behaviour of ring chromosomes, an interest that led directly to her later, more controversial work. Partly because of her sex and partly because of her exclusive commitment to research, she never obtained a tenured university position. Eventually, in 1942, she found a haven in the Carnegie Institute of Washington Laboratory at Cold Spring Harbor, where she has remained ever since.

At Cold Spring Harbor McClintock's work took a new and extremely unconventional turn. One of the legacies of her Missouri period was a chromosome segmental inversion that caused, in plants heterozygous for it, chromosome breakage at every cell division during early development (the breakage-fusion-bridge cycle). In progenies of plants that had

undergone this trauma she observed a number of unstable mutants in which inactive genes frequently reverted to activity, giving rise to clones of cells visible as spots and sectors in leaves or seeds. McClintock spent the six years between 1944 and 1950 investigating this phenomenon. She satisfied herself that the inception of instability was due, in each case, to the transposition of a movable element to the locus of the gene concerned, and that the restoration of gene activity in the spots and sectors was associated with its further

ishing results indeed. An example of the induction of high mutability at one locus by a dominant element at another locus had (though this is not mentioned by Dr Keller) already been reported by Marcus Rhoades, but the possibility of transposition was new and very difficult for most geneticists to accept. To make the problem of communication worse, McClintock added another controversial dimension to her interpretation. She had become convinced that the mutation-like and genetically controlled changes in gene activity that she was observing were representative of processes that controlled gene switching in normal development. The concept grew in her mind of a highly integrated developmental system, with a network of communications between different parts of a labile genome as well as between neighbouring segments of the same chromosome.

At the 1951 Cold Spring Harbor Symposium, McClintock outlined the main results and conclusions from her six years of intensive work. She met with almost total incomprehension. Few understood what she was saying and fewer still were prepared to accept it. Some, I am told, simply failed to see how one person could possibly have done all the work necessary to establish the conclusions that she now presented in such concentrated form.

One may get the impression from Dr Keller's account that, following this debacle, McClintock was consigned to obscurity and was rehabilitated only fifteen or twenty years later after the molecular evidence for transposable sequences in bacteria had become indisputable. This would be an exaggeration. In 1955 and 1956 she made

further attempts to convey her message (by that time complicated still further by the discovery of *Spm*, a second kind of transposable element). While these were not wholly successful, they did make some impact. At the same time, R. Alexander Brink in Wisconsin and Peter Peterson in Iowa were confirming and extending a number of McClintock's observations on transposability. Publishing their results in relatively small assimilable instalments, they convinced many previously baffled geneticists that maize transposable elements really existed. As a result, McClintock gained credibility. What Brink and Peterson did not pick up from McClintock, however, was her idea of the role of transposable elements in the integration of normal development. According



Barbara McClintock (right) in 1929, when a member of R. A. Emerson's maize genetics group at Cornell. The others (standing, left to right) are Charles Burnham, Marcus Rhoades and Emerson, with George Beadle squatting.

From *A Feeling for the Organism*, courtesy Marcus Rhoades.

transposition away from the gene locus. The first movable element to be discovered, which McClintock called *Dissociation* (*Ds*), showed its presence by causing frequent chromosome breaks at its site of residence. She was able to show that both the further transposition and the chromosome-breaking property of *Ds* were dependent on the presence elsewhere in the genome of another element *Activator* (*Ac*). This second element was autonomously transposable; in other words its transposition function could be used either for itself or in aid of *Ds* (which can now be seen as a crippled form of *Ac*).

In the context of the prevailing cytogenetical orthodoxy (an orthodoxy to which McClintock's work had previously lent so much support) these were aston-

to Dr Keller, Barbara McClintock herself thought that they had got only half of her message.

There is a strong implication in Dr Keller's account that McClintock's ideas, based on her "feeling for the organism", were all correct, and that her sceptical or uncomprehending colleagues were blinded by their devotion to successful but limited paradigms — the cytogeneticists by the supposed constancy of the genome and the molecular biologists by the "central dogma". This is disputable, at least. What Dr Keller might have attempted, but disappointingly refrains from, is an assessment of Barbara McClintock's ideas in the light of all we now know of the molecular structure of the genome. We can now see, I think, that McClintock was right both in her demonstration of transposable elements and in her conjecture about the importance of structural changes in the chromosomes as a basis for cellular differentiation. She was wrong only in the way that she linked the two ideas together. What always seemed difficult about her concept of the *controlling* role of the "controlling elements" (and it may well have contributed to the difficulty felt by the more perceptive among her audience in 1951) was the apparent contradiction between the haphazard jumps of her movable elements and the precise programming of normal development. To say that the McClintock elements represented normal controllers in a deranged state was not wholly implausible, but it hardly carried conviction.

With hindsight, we can relate McClintock's 1951 paper to three different kinds of phenomenon. First there were the transposable elements. We now know a good deal about the several kinds of movable sequences in organisms ranging from bacteria to man; and the maize elements themselves, though not yet fully characterized, seem likely to resemble in essentials some of those found in *Drosophila*. Perhaps the closest parallel is with the *P* element, responsible for the hybrid dysgenesis phenomenon in *Drosophila melanogaster*. This seems to be a molecular parasite — a good example in support of "selfish DNA", a concept that surprisingly receives no mention in this book.

The second phenomenon, which McClintock stressed as a likely parallel to the maize instabilities, was *Drosophila* position-effect variegation. This is still an unsolved problem, but it seems to have nothing to do with transposition or rearrangements of chromosome sequence. It seems rather to belong to the same category as X-chromosome inactivation in female mammals, and to involve self-perpetuating conformational states of the chromatin without change in primary sequence. The mechanism, or mechanisms, underlying such transmissible chromosomal states are not yet understood but probably soon will be. McClintock was almost certainly right in foreseeing the great importance of such

effects for cellular differentiation. Her transposable elements seem unlikely to be normal controllers of chromosomal conformational changes, but after insertion they may well modulate or at least monitor such changes. For example, both McClintock and Peterson showed that the *Spm* element in some of its locations will transmit signals or remain silent depending on the stage of development of the endosperm of the seed. We may guess that in these cases the element is responding to shifts in the state (conformation, presumably) of the chromatin domain in which it is sited.

Finally, we now have examples of kinds of normal cell differentiation that really are dependent on controlled changes in DNA sequence. Dr Keller mentions the mating type switch in yeast and likens it (misleadingly in my view) to McClintock's maize transpositions. She might equally well have mentioned the controlled deletions responsible for the differentiation of antibody-producing cells in mammals (perhaps the only aspect of mammalian cell differentiation that is so controlled), the trypanosome surface antigen switch, which has parallels with the yeast mating type system, and several

examples in bacteria and bacteriophages of reversible differentiation due to segmental inversion. All of these examples could be claimed as vindications of McClintock's idea of the controlled lability of the genome but, again, they do not suggest a controlling role for promiscuously wandering sequences such as the maize elements.

Dr Keller has written a thought-provoking book about a highly distinguished and original biologist. I think that she has succeeded remarkably well in her attempt to get inside her subject's mind, but in so doing she has lost something in objectivity. She tells us that Barbara McClintock is "proud to call herself a 'mystic'" and that she cannot always explain how she "knows" things. This is difficult to swallow. Vision, informed speculation, inspired guesswork or whatever one wants to call it has an essential place in science, but it has to be tested before it can count as "knowing". McClintock's hunches have been remarkably successful but some of them have not held up as well as others. There is no need to credit her with second sight. □

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Developing theories of evolution

Peter Lawrence

Embryos, Genes, and Evolution: The Developmental-Genetic Basis of Evolutionary Change.

By Rudolf A. Raff and Thomas C. Kaufman.

Macmillan/Collier Macmillan: 1983. Pp.395. \$34.95, £25.25.

THE THEME of this well-produced book is that "there is a genetically determined developmental program, and that evolutionary change occurs through genetic modification of this program". The authors are developmental geneticists at Indiana University and are well acquainted with both classical genetic methods and modern molecular techniques. They teach a course at Indiana which must be rather unusual, not only because competent developmental geneticists are rare birds, but also because they try to link genetics and embryology with evolution. This is a worthwhile task but a difficult one, rather like trying to make a smooth paste of diamonds and marshmallows.

I read as an interested student (the evolutionary bits) and as a specialist (the developmental genetics). In the evolutionary sections there are many interesting examples which are explained in considerable detail, yet the message can be hard to pick up. These sections are written in a tiresome, heavy style — e.g. "Noncorrespondence

between morphological and molecular phylogenies in instances in which the proteins appear to be evolving in a clocklike manner can only be caused by nonconstancy of morphological rates" — but the main problem is the organization and logic. As a student I got lost in the details, I needed more concluding sentences, more headings and generally more help with ideas. For example, the idea of "rate genes" is raised several times. They are supposed to alter the rate of one process (such as sexual maturation) without affecting the rest: they are a postulate of Goldschmidt's to whom much homage is paid by the authors. They are, I gather, an important concept. But beyond a sentence or two they are barely discussed. How would they work? What might their products be? How could they be detected? Do they exist?

The parts dealing with development and genetics are written in a clearer and more direct style. Embryology was, and is, characterized by a great deal of contentious debate and waffle, so it is pleasant to find that the authors are simplifiers and reductionists — even if they are partly in error, at least we can understand them.

They see the egg as containing a good number of carefully located determinants, each responsible for a particular developmental pathway. (The word "gradient" is only mentioned twice in the text, which must reflect a very personal view of things.) They also believe there are two fundamentally different kinds of gene; those making housekeeping products and those involved in control of morphogenesis with evolution working almost exclusively

through the latter. This is a provocative viewpoint and I think it is a valid one. Unfortunately for the argument, the evidence for regulatory genes is still scanty but the authors have done well to bring what there is together. One good example is the dramatic change in head shape that has occurred in two species of Hawaiian *Drosophila* in a remarkably short time (see illustration below). The two species can be crossed in the lab and genetic analysis suggests the differences are caused by changes at only 14–19 loci. Thus

Macroevolutionary changes in development need not be extreme. We propose that in fact the initial steps for rapid, and ultimately, large evolutionary transitions require only that key regulatory genes be few in number and accessible to nonlethal genetic alterations in their functions.

While I enjoyed the general argument, I was annoyed by the uncritical treatment of some of the material. The reader is given a one-sided story, points of controversy are not brought up, doubt not cast on doubtful interpretations. Three examples should suffice.

First, there are several pages on a "complex gene, the T-locus" of mice. As *Drosophila* geneticists, the authors should be aware that the T-locus *may* not be a locus at all, but simply a region of one chromosome arm which contains numerous unrelated genes. This region may appear to be unusual because inversions and lethals are protected from elimination by distortion of segregation (see *Nature* 299, 296; 1982).

Perhaps a greater sin is that in discussing their own work the authors can make their personal views read like facts. Consider the Antennapedia complex (ANT-C), an arbitrarily defined region of the *Drosophila* genome that contains regulatory elements. Perhaps in the hope of going places on the coat tails of E.B. Lewis's deep analysis of the bithorax complex (BX-C), ANT-C is mentioned almost every time BX-C is. We get the impression that they are very similar in structure and have comparable roles. But is this so? ANT-C is said to contain two

good candidates for regulator or selector genes (*Antp*⁺ and *Scr*⁺), the homoeotic gene *pb* and a few other genes of unknown function. Are these genes as related as the elements of BX-C are? One way to discover the function of a gene is to remove it entirely and see what happens. When this is done for the BX-C many of the segments develop identically, and this and other evidence has allowed Lewis to argue persuasively that the elements of the BX-C act individually in segments, and make them develop differently. When *Antp*⁺ and *Scr*⁺ are removed from embryos, the thoracic, labial and maxillary segments all develop as if they were prothorax (according to this book, although it is not really a clean transformation) while Struhl has shown that removal of *Antp*⁺ during subsequent development can result in antennal development in the thorax. Struhl (*Nature* 292, 635–638; 1981) has suggested that this latter result means the *Antp*⁺ gene is required to inhibit head formation in the thorax. This interpretation is not properly discussed and I think it should be, because it explains the dominant gain-of-function mutation, *Antp*.

Finally, in discussing lethal genes of *Drosophila* the authors have not mentioned the whole subject of maternal perdurance. Homozygous lethals often die when they do, not because the gene product is first, or only required then, but because they are running out of product contributed by the necessarily heterozygous mother. Thus, all or most of the products of the 1,000 genes estimated as being specific for imaginal disc development may also be necessary for the embryo. Homozygous mutants may get as far as they do because there is enough product donated by the mother to take them through embryonic development. This point should always be emphasized by developmental geneticists — it has frequently been forgotten in the past, and that led to a great deal of nonsense. □

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Reflections of cosmic discrimination

P.W. Atkins

Molecular Optical Activity and the Chiral Discriminations.

By Stephen F. Mason.

Cambridge University Press: 1982.

Pp. 273. £20, \$39.50.

OPTICAL activity has been a Hong Kong of the sciences more than once. In the times of Michael Faraday (who regarded polarized light as a "most subtle and delicate investigator of the molecular condition"), chemistry and physics most plainly overlapped where electromagnetic radiation was involved in establishing the reality and the details of molecular structure. Even the names of the contributors to the subject in its early days — Pasteur, Kelvin, Boltzmann, as well as Faraday — reflect the way it spans applications from biology to physics, with chemists in the middle busily building by clever syntheses the chiral molecules that are needed to show the effects.

In our own day the global chirality of the biological world, the handedness of the naturally occurring amino acids, remains a major problem of chemical evolution. The meeting point of the new biology and the new physics is now the speculation that the weak force is the agent of discrimination: our biochemical chirality may be (if it is not merely fortuitous) a reflection of the cosmic chirality of matter.

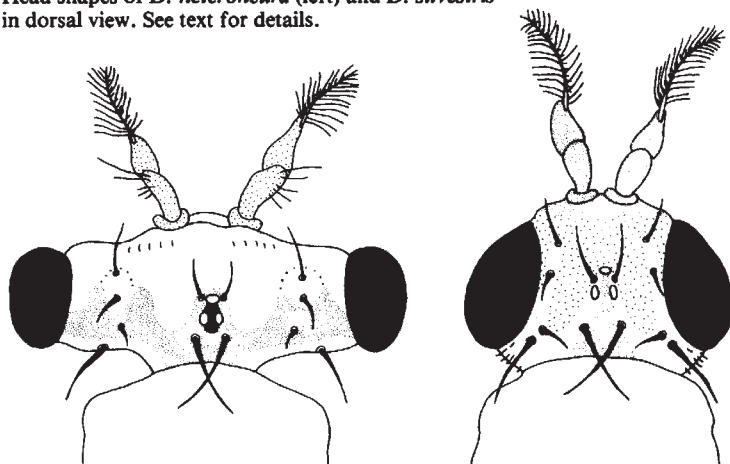
Professor Mason's book spans this world. He takes us from its historical origins up to our present fascination with our own chirality. The topics include the nomenclature of chiral systems, mechanisms of optical activity, octant rules, polymeric and inorganic transition metal systems, vibrational optical activity (an increasingly important structural technique) and the varieties of chiral discrimination, including those depending on different rates of reaction as well as on subtle differences of energy.

This is very much a practitioner's book, for the emphasis throughout is on experimental information and technique. It is also very much a book for theoreticians to turn to, in order to find at least a brief discussion of what is now known. The style is condensed, and the text is more an extended review than a relaxed introduction. But the aspiring professional, at whom it is aimed, will find much to reward a slow and thoughtful reading. □

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● John Lenihan's *Science in Action*, first published in 1979, has recently been issued in paperback by Adam Hilger. Price is £4.95.

Head shapes of *D. heteroneura* (left) and *D. silvestris* in dorsal view. See text for details.



From *Embryos, Genes, and Evolution* (re-drawn from Val, 1977).

Geochemistry: up and down and all around the oceans

Henry Elderfield

Tracers in the Sea.

By Wallace Smith Broecker
and Tsung-Hung Peng.

*Lamont-Doherty Geological
Observatory, Columbia University:
1983. Pp. 690. \$35.*

THE past decade will be judged as crucial in the development of chemical oceanography. We now know that hydrothermal circulation of sea water through ocean ridges has a profound effect on the geochemistry of elements in the oceans, rivaling continental weathering processes in its contribution to the dynamics of element transport across ocean boundaries. Technical advances in studying trace metal and isotope distributions in ocean waters, in measuring chemical fluxes in particulate materials and in studying the chemistry of pore waters have led to a greater awareness of the flow of elements within the marine environment.

Further, work using natural and anthropogenic radioactive tracers has added much to our understanding of the movement of ocean waters; in particular, the chemical tracers offer the most important tool we have for studying the oceanic thermocline. Advances have also been made in documenting the chemical history of sea water — especially glacial to interglacial changes over the past 10⁵ years and long-term changes related to plate movements and crustal weathering — although serious problems still exist in deciphering the sedimentary record. Man is also taking a hand in changing the oceans' chemistry, and urgent efforts are being made to evaluate the impact of fossil-fuel carbon dioxide on the oceans' chemical cycles.

It is also a decade since the publication of W.S. Broecker's *Chemical Oceanography* (Harcourt Brace Jovanovich, 1974), a lively, stimulating, arm-waving book and the predecessor of *Tracers in the Sea*. In the new book, co-written with T.-H. Peng, Broecker blends an account of these aforementioned advances with some material from the old to produce a major text. Like its predecessor, the book stimulates by generalization and speculation, and by addressing the explanation rather than the documentation of how the oceans operate chemically.

A principal theme is the Earth's carbon cycle and a major data set used is that produced by the American GEOSECS (Geochemical Ocean Sections) programme, the success of which was due in large part to Arnold Bainbridge (to whose memory the book is dedicated). The book

explores the links between carbon in the atmosphere, sea water and oceanic sediments, showing how this focal element in large part controls the chemistry of sea water and the distribution of sediment types, and how perturbations to carbon sources can be linked to past and future changes in oceanic chemistry. It provides, too, a topical summary of how chemical tracers give key information on water movements. Carbon isotopes play a central role in such studies: carbon-14, together with the nutrients silicate and phosphate, and tritium, radon-222, radium-228, helium-3 etc., to trace the movements of water through the deep sea and the oceanic thermocline; carbon-13 to trace differences in phosphate between water masses, thus permitting reconstructions of ocean chemistry in glacial times; and carbon-13 and carbon-14 to constrain and refine models of the oceans' uptake of carbon dioxide produced by the burning of fossil fuels.

Wrapped around this account of the marine carbon cycle are data and ideas emphasizing the chemical dynamics of the

oceans and the means for investigating them: the uranium and thorium decay chains for determining mixing and accumulation rates; lead and neodymium isotopes as source and reactivity tracers; excess helium as a hydrothermal tracer; and so on.

Overall, this leads to a very stimulating, reasonably integrated but necessarily focused view of what controls distributions of chemicals in the sea. To guide the reader through the various concepts are twelve anagrammatic apostles who, sharing the authors' well-known propensity for box models, lurk at the conclusion of each chapter and ask for advice on the interpretation of results from their aquaria experiments. Anyone who reads this successful book, parochialisms and all, will learn a lot about the chemical driving force in the oceans and will appreciate much of the interplay of ocean physics and biology with chemical cycles that makes marine geochemistry so fascinating and challenging. □

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Why study H-Y?

Peter Goodfellow

H-Y Antigen and the Biology of Sex Determination.

By Stephen S. Wachtel.

*Academic/Grune and Stratton: 1983.
Pp. 302. \$44, £29.20.*

THE mammalian Y chromosome is required for male sexual determination. As it is almost totally lacking in defined genetic loci, any gene found on the Y chromosome has been regarded as potentially related to sexual determination.

Male-specific antigens have been defined by tissue transplantation, *in vitro* T-cell killing and by serological assays. The antigen or antigens recognized by these disparate assays are all referred to as 'H-Y antigens' or 'H-Y'. Transplantation, T-cell killing and, initially, serological assays showed a correlation between the presence of the H-Y antigen and the Y chromosome, and for this reason H-Y antigen has been extensively studied as a candidate for the elusive male determining substance. Subsequent work with the serologically defined antigen, particularly on H-Y antigen positive XO individuals, has relegated the Y-linked gene from a structural to a controlling locus. The importance of the H-Y antigen in sex determination now rests on a series of *in vitro* functional blocking tests based on the morphological evaluation of aggregation of germ cells.

Stephen Wachtel is well qualified to write the history of the H-Y antigen and sex determination as he has been responsible for much of the work in this area. Two of

the difficulties in writing scientific tomes is to make the work interesting to non-specialists and to translate the precise scientific words (jargon) into a more generally understood English. Dr Wachtel has solved the first problem by spicing his book with quotations from antiquity and from the more prescient of contemporary scientists. The second problem has only been partly solved: the immunology is explained clearly but the medical descriptions of the unfortunate individuals with indeterminate sexual phenotypes requires close study of *Gray's Anatomy* and a medical dictionary before understanding dawns.

However, the final test of a scientific book must be the scientific content. The review of the H-Y antigen literature is exhaustive and gentle; differences in opinion or results are nearly always reconciled by generous interpretation. In my view a more critical approach would have been ultimately more constructive as it is difficult for outsiders to form conclusions from the baroque serological experiments needed to define the H-Y antigen status of an individual. For example, it is not clear to me if normal females express a basal level of serologically defined H-Y antigen.

It was a monumental task to sort through all the papers on H-Y antigen and Dr Wachtel's book is worth buying as a compendium of that information. But the book will not be the basis of a consensus on the nature of the H-Y antigen and its relationship to sex determination. The last word remains to be written. □

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